Regulation of Auxin Transport by Modulatory Drugs and Protein Phosphorylation

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To my Parents, Ted Buff and Tobi.

1 SUMMARY

Several only partially understood mechanisms provide plants a sophisticated plasticity to respond to environmental changes by reprogramming their developmental and cellular responses. This ability is very often regulated by auxin – generally considered as a potent phytohormone - via the establishment of highly regulated concentration gradients. In agreement with the chemiosmotic model, a complex regulatory network of in- and efflux carriers and transporters together with interacting proteins control the direction and capacity of auxin transport.

Although several proteins of the polar auxin transport (PAT) machinery are identified, little is known about the regulatory mechanisms that affect auxin transport activity directly. In fact auxin can be regulated by mechanism ranging from transcriptional gene regulation to a variety of post-transcriptional modifications. Additionally, a group of naturally occurring compounds, the flavonols, provide a variety of different modulatory abilities to fine-tune auxin distribution on various molecular levels. In this respect the present work reflects the huge variability of auxin regulatory mechanism on the basis of three projects each representing a different modulatory component of the auxin transport machinery.

In my first project, we showed in a collaboratory work the effect of flavonols, especially quercetin, on gene expression of the auxin efflux carrier, PIN1, and as a consequence on the redistribution of auxin. Vectorial root bending is mediated by PIN2, a member of the PIN-FORMED auxin efflux carrier family. Defects in the gravitropic response in the *pin2* mutant background could be linked to an altered flavonoid accumulation in the elongation zone of the root tip. Detailed analysis revealed that low concentrations of flavonoids induce the ectopic expression of *PIN1* to replace PIN2 in the appropriate tissue in order to rescue the gravitropic response. Our finding indicates that flavonoids do not solely inhibit efflux transporters, but also act as modulators of gene expression of a PIN protein that finally leads to changes in plant growth responses (Santelia et al., 2008).

A second part of my work characterizes a novel auxin transport inhibitor, BUM, that was identified by a chemical genomics approach. A detailed analysis of its effect on plant physiology and further molecular targets demonstrates that BUM affects the auxin transport machinery directly by interaction with the ABCB-type auxin transporters. Here, modification of transporter activity leads to changes in auxin movements and, as a consequence, to different plant responses. BUM acts in some respects similarly to NPA (N-1-naphylphtalamic acid), a well-known auxin transport inhibitor, but is active at much lower concentrations (Kim, Henrichs 2009, under revision). As a consequence, detailed studies of NPA and also BUM and its site of action helps to bring new insights into the biochemistry and physiology of polar auxin transport and ABCB functionality.

As a starting point of the third project of my PhD, we identified in a tandem affinity purification approach the AGC kinase PINOID (PID) as a TWISTED DWARF1 interacting protein. It has been shown that PIN protein phosphorylation by PID affects their polar localization and thus the direction of auxin transport. Furthermore TWD1 directly interacts with ABCB1, a primary active auxin transporter that also resembles a well-known target of phosphorylation dependent regulation. Therefore, we

aimed to investigate the direct effect of protein phosphorylation events on the regulation of protein transport activity. Interestingly, co-expression of PID with representative members of the ABCB- or PIN family in tobacco cells, leads either to an enhanced transport activity or to a complete inhibition. Importantly, signaling pathways upstream of PID have the capacity to modulate the transport-dependent distribution of auxin by affecting the phosphorylation status. We hypothesize that modulatory drugs, like flavonoids, which are used as clinical kinase inhibitors, act on the phosphorylation dependent regulation of auxin transport. Detailed binding studies revealed PID as a direct target for quercetin suggesting quercetin as a negative regulator for PID activity.

Taking all this into account, this work contributes to a detailed understanding of the regulatory mechanism of auxin transport on different molecular levels. The modulatory impact of flavonoids on auxin transport might result from a combinatory effect on ABCB activity and its interaction with TWD1 and additionally on PIN gene expression and cellular trafficking. Additionally, investigation of phosphorylation dependent regulation of membrane transport contributes important information about general cellular regulatory mechanisms that are conserved in eukaryotic cells. Flavonoids, as endogenous plant compounds, are also of interest for the medical research field and are widely used as clinical drugs. So far the molecular targets of quercetin during auxin transport were not clear. Our analysis of the sites of quercetin action provides new insights into the mechanisms and potential of naturally occurring drugs.

2 ZUSAMMENFASSUNG

Pflanzen besitzen eine erstaunliche Plastizitzät - ein Phänomen, welches bis heute nur ansatzweise verstanden wird. Sie sind in der Lage sich wechselnden Umweltbedingungen aktiv anzupassen, indem sie ihr entwicklungsbiologisches Programm auf der zellulären Ebene umprogrammieren. Diese Fähigkeit wird hauptsächlich durch ein multifunktionelles Phytohormon, names Auxin reguliert, welches durch komplexe Regulationsmechanismen Konzentrationsgradienten aufbaut um situationsbedingt agieren zu können. In Anlehnung an das Chemiosmotische Model bilden In- und Efllux Carrier und Transporter zusammen mit interagierenden Proteinen ein ausgeklügeltes regulatorisches Netzwerk, um die Richtung und Kapazität des Auxintransports zu regulieren.

Obwohl schon einige Proteine des Auxintransport-Komplexes entdeckt wurden, ist noch relativ wenig über die regulatorischen Mechanismen bekannt, welche die Aktivität des eigentlichen Transportes beeinflussen. Der polare Auxintransport kann von einer Vielzahl verschiedener Mechanismen kontrolliert werden, die auf transkriptioneller oder post-transkriptionelle Ebene agieren. Die Flavonoide, eine Gruppe von natürlich vorkommenden pflazlichen Sekundärmetaboliten, können den Auxin Transport ebenfalls auf verschiedenste Weise beeinflussen, meist um die genaue Verteilung in einem entsprechenenden Gewebe zu gewährleisten. In Anlehnung, repräsentieren die drei Projekte meiner Doktorarbeit anhand verschiedener Beispiele die ungeheure Vielfalt der regulatorischen Mechanismen des zellulären Auxintransports.

In Zusammenarbeit analysierten wir in meinem ersten Projekt das Flavonoid Quercetin und dessen regulatorischen Einfluss auf die Gentranskription von PIN1, einem Auxin Effluxprotein, und dessen Auswirkungen auf die Auxin Verteilung in der Wurzelspitze. PIN2, ein weiteres Protein der PIN-FORMED (PIN) Efflux-Carrier Familie, reguliert die Orientierung der Wurzelspitze und dadurch das Wurzelwachstum in eine bestimmte Richtung. Die mangelnde Fähigkeit der *pin2* Keimlinge sich entsprechend der Schwerkraft zu orientieren wird unter anderem einer veränderten Flavonoidzusammensetzung in der Streckungszone der Primärwurzelspitze zugeschrieben. Detailierte Analysen zeigen, daß die Verabreichung geringer Mengen von Flavonoiden zu einer Reorientierung der Wurzelspitze führt und somit ein Wachstum entlang des gravitropen Vektors erneut gewährleistet. In diesem Fall wird das fehlende PIN2 Protein durch PIN1 ersetzt, welches nun in dem entsprechenenden Gewebe funktionell exprimiert wird. Unsere Erkenntnis zeigt demnach, das Flavonoide nicht nur den Auxintransport direkt beeinflussen können, sondern auch als Modulatoren der Gentranskription in Erscheinung treten, um das Pflanzenwachstum nachhaltig zu beeinflussen (Santelia et al., 2008).

In meinem zweiten Projekt haben wir einen neuen Auxintransport--Inhibitor namens BUM, charakterisiert, welcher in einem *chemical genomics screening* entdeckt wurde. Die genauen Untersuchungen der Wirkung auf die Pflanzenphysiology und die entsprechenenden molekularen Analysen haben ergeben, dass BUM direkt auf den Auxin Komplex wirkt, indem es mit ABCB1, einem ABC Transporter, interagiert. Die verursachten Modifikationen beeinflussen den Auxin Transport und haben damit weitreichende Konsequenzen für das gesunde Pflanzenwachstum. BUM ist dem weit

verbreiteten Auxin Transport Inhibitor NPA (N-1-naphylphtalamic acid) in gewisser Weise ähnlich, allerdings wirkt es schon bei dreissig-fach geringerer Konzentration (Kim, Henrichs 2009, under revision). Die detaillierte Untersuchung von BUM (und auch NPA) helfen somit, neue Erkenntnisse über die Biochemie und Physiologie des polaren Auxin Transports und die Funktionen von ABCB-Transportern zu gewinnen.

Zu Beginn meines dritten Projekts, haben wir durch einen *tandem affinity purification* TAP) Ansatz die AGC Kinase PINOID (PID) als TWISTED DWARF1 (TWD1)-interagierendes Protein identifiziert. TWD1 interagiert ausserdem direkt mit ABCB1, einem primär aktiven Auxintransporter, welcher bekannt ist für seine prominenten Phosphorylierungsstellen. Daraus hat sich für uns die Frage ergeben, ob PID einen regulatorischen Einluss auf die Transportaktivität der Proteine hat. Interessanterweise führte die Koexpression von PID in Tabakprotoplasten mit jeweils einem ABCB- oder einem PIN Protein zur Stimulation oder aber auch zur absoluten Inhibition des Auxintransports. Regulatorische Einflüsse welche auf PID wirken, beeinflussen schlussendlich die Auxinverteilung. Zusätzlich wollten wir die RThese überprüfen, ob Flavonoide als Modulatoren und Kinaseinhibitoren, den Auxintransport durch Regulation der Phosphorylierung beeinflussen können. Detaillierte Bindungsstudien haben ergeben, dass Quercetin in der Tat die PINOID Kinase bindet und dadurch negativ reguliert.

Grundsätzlich bietet diese Arbeit neue Einblicke in regulatorischen Mechanismen des polaren Auxintransports auf verschiedenen molekularer Ebenen. Die modulatorischen Einflüsse der Flavonoiden zeugen wohl von einem kombinierten Effekt, da sie sowohl ABCB-Transporter und das interagierende Immunophilin, TWD1, modulieren, als auch die Genexpression von PIN Proteinen beeinflussen. Darüber hinaus dürften unsere Erkenntnisse zum Mechanismus der Proteinphosphorylierungen allgemein nützliche Informationen zum Verständnis zellulärer Vorgänge in Eukaryoten liefern. Flavonoide sind ausserdem interessant, da sie als natürlich vorkommende Stoffe eine nützliche Quelle für mögliche Medikamente darstellen. Der molekulare Effekt von Quercetin war bis heute unbekannt. Somit trägt unsere wissenschaftliche Analyse einen Teil dazu bei, Quercetin als natürliches Produkt für medizinische Zwecke attrativ zu machen.

3 INTRODUCTION

Good communication leads to success (Def: Wikipedia). Therefore, plants have developed specific mechanisms to communicate not only with each other, but also to transfer information from one tissue to another. A complex network of different plant hormones (phytohormones) and other chemical signals is necessary for the regulation and coordination of metabolism, growth, and morphogenesis of a plant organism. Generally, (phyto-)hormones are signaling compounds that act at very low concentrations and seem to function mainly in the tissue in which they are produced (autocrine). For most phytohormones the separation between tissues of hormone biosynthesis and hormone action, which is typical for animal hormones, is less sharp. However they are widely addressed as hormones, a term that is also used in the following text. Today the most intensively studied plant hormone is auxin, which fulfills an important role in regulating the sophisticated mechanism of developmental plasticity to respond to environmental changes and to function in patterning and organogenic processes (Figure 1). Its particular characteristic to move in a directional manner from cell-to-cell to create local maxima will be addressed in detail in the following work.

3.1 Auxin – The Power of Movements in Plants

“A chemical messenger is responsible for the formation and growth of different plant organs and external factors such as gravity could affect the distribution of these compounds within the plant”. This anticipatory idea originated already in 1865 from Julius von Sachs (1832-1897) and marks the beginning of the auxin history (Sachs, 1865). Fifteen years later Charles Darwin and his son Francis presented in their book "The Power of Movement in Plants" studies on plant growth and the phenomenon of phototropism on seedlings of canary grass (*Phalaris canariensis*). They claimed that an “influence is transported” that causes bending of the seedling towards light (Darwin and Darwin, 1880). But the identity of the mysterious compound was still unclear. It was in 1926 when a graduate student from Holland by the name of Fritz Went published a report describing that an active growth-promoting chemical can diffuse into a gelatin block, which promotes growth when placed on a coleoptile section. At the end auxin, namely **IAA** (Indole-3-acetic acid, Figure 1) was the first plant hormone discovered (Thimann, 1938) and due to its ability to promote plant growth the name auxin, from the greek auxein “to grow” was born. Since then additional natural auxins have been identified like 4-chloro-indole-3-acetic acid, indole-3-butyric acid (**IBA**) and phenylacetic acid (**PAA**), with the ability to mimik IAA-like physiological activity (Pennazio, 2002). A comparison of compounds that possess auxin characteristics suggest that the charge separation of 0.5 nm of the negative carboxyl group on the side chain from a positive charge on the ring structure may be an essential structural requirement for auxin activity (Plant Physiology, Edition 2008).

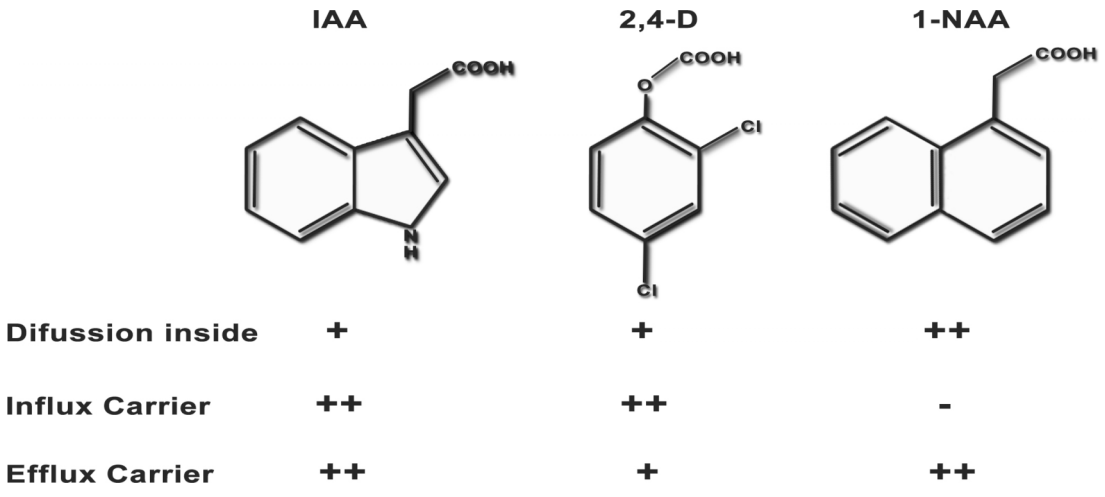


Figure 1. Structure of some synthetic auxins

Indole-3-acetic acid (IAA) is considered to be the most important natural auxin, 1-naphthalene-acetic acid (1-NAA) is a horticulturally important auxin and 2,4-dichlorophenoxyacetic acid (2,4-D) is a common selective herbicide.
++ strong; **+** weak; **-** no effect on cellular accumulation.
(Based on Delbarre et. al. 1996, Marchant et.al. 1999, modified from Taele et al. 2006).

A wide range of synthetic auxins is also available and most of them are used as herbicides in agriculture. **2,4-D** (2,4-dichlorophenoxyacetic acid), one of the world's most widely used weed-killers, is a membrane-impermeable auxin, which requires active up-take to get into the cell and is poorly exported. Additionally, **1-NAA** (1-naphthylacetic acid), but not the anti-auxin 2-NAA, can freely diffuse over the plasma membrane into the cell and resembles an excellent substrate for active efflux (Figure1).

Although its chemical structure is relatively simple, auxin plays a crucial role in regulating and coordinating plant growth and is involved in mainly all developmental processes, including directional growth responses (tropisms) (Esmon et al., 2005), (Palme et al., 2006), control of plant architecture (Bainbridge et al., 2008; Dubrovsky et al., 2008), abiotic and biotic stress responses (Berger, 2002; Spoel and Dong, 2008) and flower and embryo development (Aloni et al., 2006; Friml, 2003). The individual effect depends on the auxin availability and concentration in the according tissue of the plant.

In this work the following concepts are mostly based on studies in the model plant *Arabidopsis thaliana*. However, they seem to apply to general concepts in all plants.

3.2 Auxin Synthesis, Degradation and Storage

The plasticity of plant development is regulated by the differential distribution of auxin between cells and tissues in order to create local auxin maxima or gradients (Cheng et al., 2006, 2007a). But before we can fully understand the sites of auxin activity, it is vital to recall where auxin is synthesized in the plant. IAA biosynthesis occurs in rapidly dividing and growing tissues, although it has long been held that auxin is predominately produced in young developing leaves. In fact, local auxin biosynthesis contributes to a significant amount of active auxin (Cheng et al., 2006, 2007a; Palme and Nagy, 2008; Stepanova et al., 2008; Tao et al., 2008). Specially, in the root meristem local auxin biosynthesis together with transport mechanisms assemble an auxin maximum that is important for root development and architecture (Petersson et al., 2009). Similarly, random stimulation of auxin biosynthesis in a single root pericycle cell causes a localized increase in auxin that is sufficient to induce the initiation and formation of lateral roots from competent cells (Dubrovsky et al., 2008). Interestingly, a novel concept of auxin action reveals that in the flower an auxin minimum is important for differentiation of a small number of carpel cells (Sorefan et al., 2009). These studies indicate that the auxin concentration in a given cell is capable of modifying its developmental program.

Thus far, one tryptophan (Trp)-independent (Ostin et al., 1999) and four Trp-dependent pathways for the IAA biosynthesis have been proposed (Figure 2). These are the indole-3-acetamide (**IAM**) pathway, the indole-3-acetaldoxime (**IAOx**) pathway, the tryptamine (**TAM**) pathway, and the indole-3-pyruvic acid (**IPA**) pathway reviewed in Chandler, 2009; Woodward and Bartel, 2005. Clearly, these multiple redundant pathways are not always completely discrete and often converge, but parallel auxin synthesis provides a well-buffered system, potentially flexible to many points of regulation and modulation in different tissues and in response to environmental changes. Only the TAM and IPA pathways have been highlighted as relevant to development *in planta* and it is still an open question if they represent independent or partially overlapping functions for auxin production (Delker et al., 2008). The recent characterization of the flavin monooxygenase-like enzymes of the **YUCCA** family (YUC) showed developmental defects when mutated in Arabidopsis plants. They catalyze the hydroxylation of tryptamine and overexpression of YUC1 (Zhao et al., 2001) and YUC6 (Kim et al., 2007a) leads to phenotypes characteristic of auxin overproducing mutants (Figure 2d).

The majority of IAA in the plant is in the form of inactive conjugates, which can be stored or transported (Figure 2f). The main storage method is the conjugation of IAA to sugars, amino acids or peptides to render it inactive (Rampey et al., 2006). Although IAA conjugates are generally considered as inactive metabolites, a new finding reveals previously unrecognized auxin regulatory mechanisms for **IAA-Trp** (Tryptophan) conjugates. Apparently IAA-Trp acts as a super-potent endogenous inhibitor in order to antagonistically regulate auxin responses specifically during primary and lateral root growth (Staswick, 2009). In addition, IAA can be methylated or converted to indole-3-butyric acid (**IBA**). These are reversible reactions and free IAA can be released when it is needed (Woodward and Bartel, 2005). Irreversible degradation of IAA occurs by modification of the indole ring and its side chains by IAA oxidases. As a concrete example, local auxin synthesis combined with degradation/conjugation is

important for female gametophyte development (Pagnussat et al., 2009).

Auxin degradation pathways are themselves regulated by the amount of auxin. For example genes encoding for members of the **GH3**-like protein family are able to conjugate IAA to several amino acids including Trp (Figure 2f). They are highly auxin-inducible (Hagen and Guilfoyle, 2002) and the auxin catabolism is promoted by elevated IAA level to prevent excessive amounts of free auxin (Staswick et al., 2005). Therefore auxin storage and degradation contribute significantly to the active auxin pool.

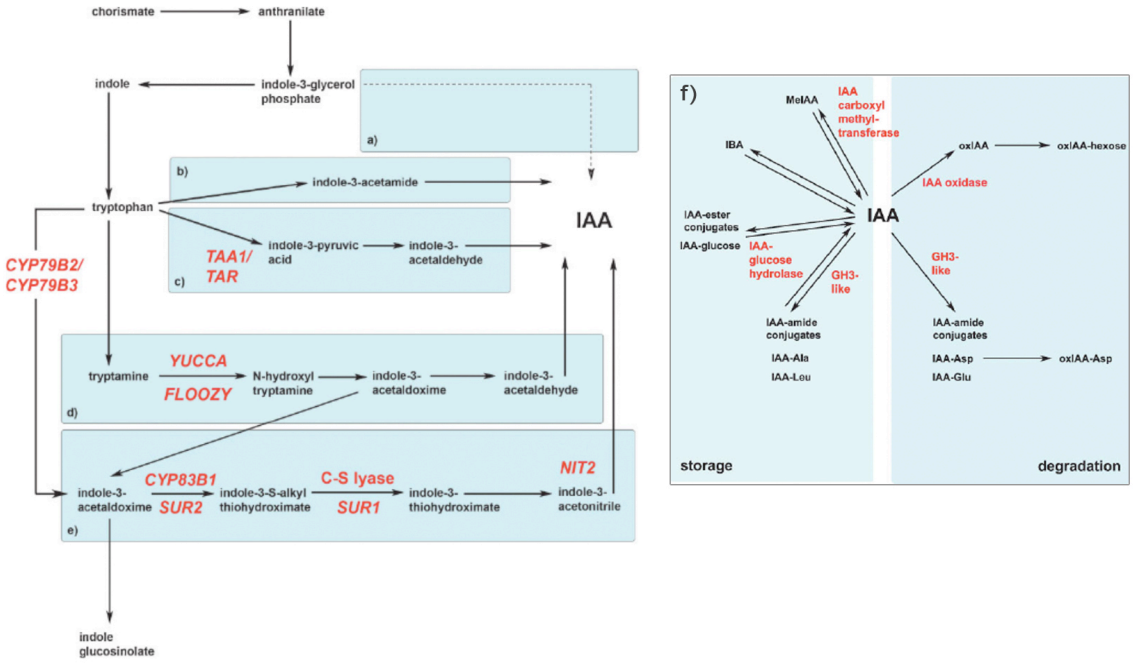


Figure 2. Different auxin biosynthesis pathways

a) The postulated tryptophan (Trp)-independent pathway. In the four main branches of Trp-dependent pathway, IAA is synthesized from indole precursors via, **b**) indole-3-acetamide (IAM), **c**) indole-3-pyruvic acid (IPA), **d**) tryptamine (TAM) or **e**) indole-3-acetaldoxamine (IAOx), **f**) summary of main storage and degradation pathways for IAA. The positions of enzymes encoded by genes that result in a phenotype when mutated are shown in red. (Modified from Chandler 2009)

3.3 Cellular Auxin Signaling

The transcription of mainly all known proteins of the auxin transport machinery (ABCBs, PINs, PID, AUX1/LAX) is influenced by auxin (Geisler et al., 2005; Noh et al., 2001; Terasaka et al., 2005; Vieten et al., 2005) via signaling through a cascade including the F-box protein transport inhibitor response (**TIR1**) auxin receptor. The key players in auxin signaling are the auxin receptors of the TIR1/AFB (Auxin-related F-Box proteins) family, the auxin signaling repressors of the **AUX/IAA** family and the transcription factors of the **ARF** family (Figure 3) (Delker et al., 2008).

Auxin Response Factors (ARFs) are the main transcriptional regulators of auxin regulated gene expression (Hardtke et al., 2004) by binding to a consensus auxin-responsive element (ARE). AUX/IAAs, which are degraded in an auxin-dependent manner, negatively regulate auxin signaling by binding and repressing the ARFs. Importantly, different sets of ARFs and Aux/IAAs are produced in a given tissue, thereby generating a certain combination that in turn regulates transcription of a particular set of auxin-responsive genes.

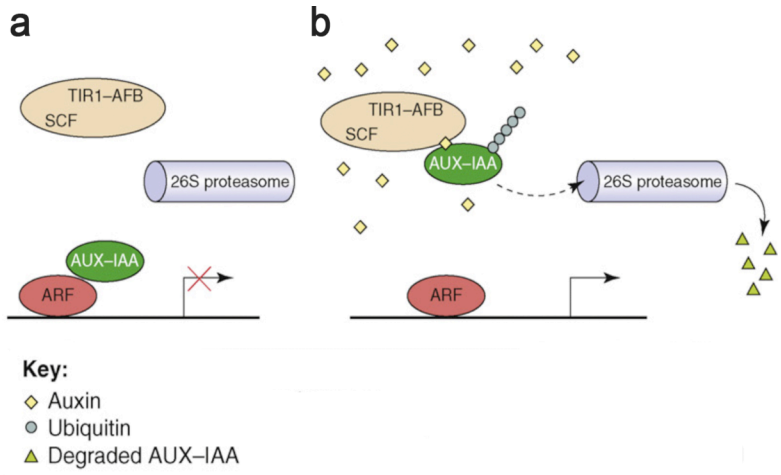


Figure 3. Auxin signaling (modified from Lau et al., 2009)

In the absence of auxin, AUX/IAAs repress ARF activity (Figure 3a). In the presence of auxin, Aux/IAAs are recruited by $SCF^{TIR1/AFB}$ (Skp1-cullin-F box protein) and interact directly with TIR1. This leads to ubiquitination of Aux/IAAs and as a consequence to protein degradation by the 26S proteasome. TIR1 binds auxin at physiologically relevant concentrations (K_d 20-80nM) and this binding stimulates the interaction between TIR1 and Aux/IAAs (Dharmasiri et al., 2005; Kepinski and Leyser, 2005). The ARFs are released from repression and induce gene expression (Figure 3b).

However, a more rapid $SCF^{TIR1/AFB}$-independent signaling pathway was also suggested and a good candidate for an additional auxin receptor protein is the auxin binding protein1 (**ABP1**) (Hertel et al., 1972; Lau et al., 2008; Yamagami et al., 2004).

3.4 Auxin Transport

Although local auxin biosynthesis significantly contributes to a stable auxin distribution (Petersson et al., 2009), this mechanism alone cannot account for a constant auxin gradient. A nice example of the correlation between local biosynthesis and auxin transport was just recently published. Alteration in planar polarity for root hair development involves local auxin synthesis and its redistribution by influx and efflux carriers (Ikeda et al., 2009).

In general, young shoot tissue is biosynthetically highly active and from there auxin can be distributed throughout the plant by two different transport ways, varying in celerity and directionality (Figure 4). In principle auxin uses the fast but passive, long-distance source-to-sink transport through the mature phloem (Galweiler et al., 2000; Galweiler et al., 1998; Scarpella et al., 2006) and the slow, directional cell-to-cell directional transport that is highly regulated. The latter transport mechanism is unique for auxin and has been studied intensively (Petrasek and Friml 2009). These two pathways seem to be connected at the level of phloem loading in leaves (Marchant et al., 2002) and phloem unloading in roots (Swarup et al., 2001).

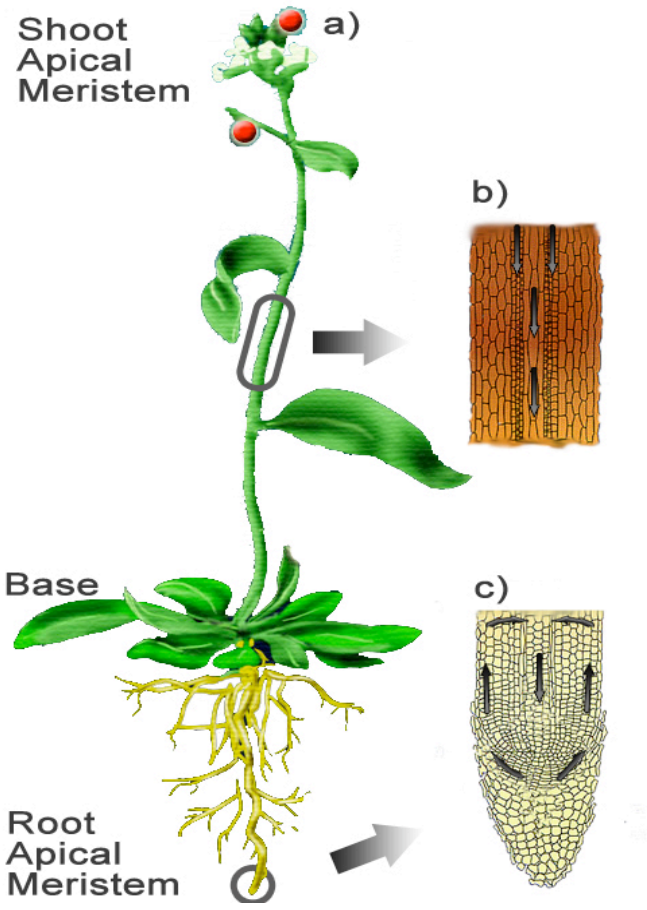


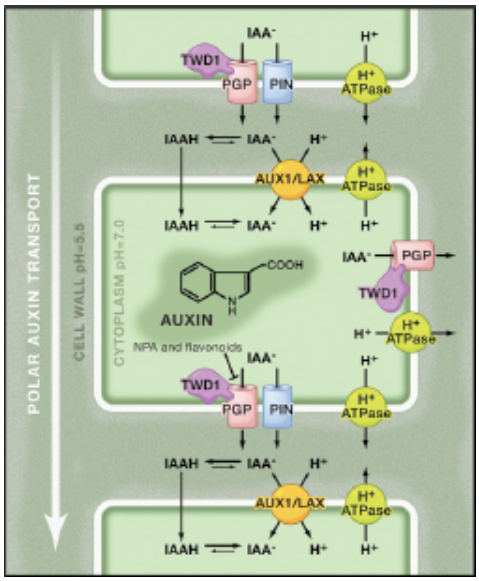
Figure 4. Auxin transport directions and sites of biosynthesis

a) Auxin is primarily synthesized in the shoot apical meristem (SAM) or in young developing leaves (red dots), from here auxin is distributed throughout the plant. **b**) It travels basipetally through the stem. **c**) In the root stele auxin moves acropetal from the base in to the root apical meristem (RAM) and reverts basipetally in lateral root cap and epidermal cells. Finally auxin builds a stable reflux system in root tip.

Although the vasculature-based transport has a major impact on auxin distribution, this work mainly concentrates on understanding the mechanisms behind the auxin transport from cell to cell. Basically, this process is mediated by the transport activity and asymmetric (polar) localization of specific influx and efflux carriers/transporters in the plasma membrane. For the following work it is important to define the different types of efflux and influx proteins. Throughout this work, primary active transporters probably transporting one molecule IAA per ATP hydrolysis (like ABC transporters) are referred to as transporters. Secondary active symporters co-transporting $2H^+$/IAA (like AUX1) and permeases with so far unclear stocheometry and mechanism are referred to as carriers.

3.4.1 The Chemiosmotic Model

Before we discuss in detail the mediators of the PAT machinery it is important to define the mechanisms underlying directional auxin transport. Basically, the chemiosmotic model (Rubery and Sheldrake, 1973) postulates that pH differences between the extracellular space and the cytoplasm is the basis of the transcellular transport of auxin (Figure 5). IAA is a weak acid (pKa=4.75) and about 15% of the IAA pool becomes protonated in the relatively acidic (pH 5.5.) environment of the extracellular space, the apoplast. In this lipophilic form IAA can diffuse passively over the plasma membrane into the cell. This passive influx is supplemented by the action of auxin influx carriers, like the $2H^+$ co-transporter **AUX1** (Bennett et al., 1996a; Swarup et al., 2008). In the more neutral pH of the cytoplasm, IAA turns into its deprotonated, membrane-impermeable form and is trapped inside. To exit the cell it must be transported actively over the plasma membrane by specific auxin transporters and carriers, which direct IAA according to their status of transport activity and membrane localization. Twenty years after the postulation of the chemiosmotic model molecular support followed. The first polarly localized auxin efflux carrier **PIN1**, a member of the PINFORMED protein-family, was identified (Galweiler et al., 1998). But in nature it is rarely the case that a complex process is mediated by only one component and, as predicted, efflux transporters are identified to also contribute to a stable auxin distribution. **ABCB/PGP** proteins control ATP-dependent auxin transport over the plasma membrane (Geisler et al., 2005) while the PIN carriers determine the directionality of auxin transport by means of their polar subcellular localization (Friml, 2003). These components will be discussed in more detail later in this chapter.



As a side note, it is not clear how auxin avoids the symplastic transport through the plasmodesmata, whose diameter could easily gather several auxin molecules. This suggests the presence of an active mechanism that blocks the auxin runaway through the plasmodesmata (Samaj et al., 2002) and implies functional advantage for using the transcellular pathway through the apoplast.

Figure 5. The chemiosmotic model for polar auxin transport

The low pH in the apoplast is maintained through the activity of plasma membrane H+ ATPases. In the relatively acidic environment, a fraction of the weak acid, indole-3-acetic acid (IAA), becomes protonated. The protonated (IAAH) form is more lipophilic and can diffuse freely over the plasma membrane into the cell. Auxin is also actively taken up from the apoplast by H+/IAA symport mediated by AUX1/LAX influx carriers. In the cytosol, it dissociates and gets trapped inside the cell in its deprotonated form (IAA–). IAA– can exit cells by the action of ABCB- or PIN-type efflux carriers. The polar cellular localization of the carriers determines the directionality of the intercellular auxin flow. ABCB activity can be modulated by 1-naphthylphthalamic acid (NPA) and flavonoids that interfere with the interaction of ABCB and a protein that regulates it, TWISTED DWARF1. (From Vanneste and Friml 2009).

3.4.2 The Auxin Transport Machinery

3.4.2.1 AUX1/LAX Influx Proteins

Although IAA in its deprotonated form can diffuse passively over the plasma membrane, the protonated auxin anions (IAA^-) are delivered into the cell by members of the conserved AUXIN RESISTANT1/LIKE AUX (AUX1/LAX) protein family which act as $2H^+$ co-transporters (Figure 5) (Bennett et al., 1996a; Swarup et al., 2008). The *Arabidopsis* genome encodes for four putative auxin influx carriers: *AUX1* and the *LAX1*, *LAX2*, and *LAX3* (Parry et al., 2001). All are involved in phyllotaxis (Bainbridge et al., 2008) and AUX1 together with LAX3 controls lateral root development (Swarup et al., 2008).

The AUX1 protein was originally identified through characterization of the *Arabidopsis auxin1* (*aux1*) mutant, which was isolated from a mutant screen for roots resistant to the membrane-impermeable synthetic auxin 2,4-D (Pickett et al., 1990). Importantly, the selective response of *aux1* roots towards 2,4-D versus 1-NAA, which is freely membrane permeable, diagnoses an impaired hormone uptake (Figure 1). Experiments in *Xenopus laevis* oocytes and *S.pombe* demonstrate auxin and 2,4-D import when AUX1 or LAX3 were heterologously expressed (Swarup et al., 2008; Yang and Murphy, 2009; Yang et al., 2006). AUX1 localizes to the plasma membrane of various cell types and especially in young phloem cells (protophloem), AUX1 targets to the upper plasma membrane, presumably in order to unload auxin from the phloem into the short-range transcellular transport pathway in the root tip (Dharmasiri et al., 2006). The binding of IAA to AUX1 (K_d of 2.6µM) was determined by Carrier et al. 2008, which can be displaced by auxin analogues (2,4-D, 1-NAA) and auxin influx inhibitors. In summary, there is compelling biochemical and genetic evidence that the AUX1/LAX proteins act as influx carriers and have important roles in root growth, tropisms and organogenesis (Figure 5).

3.4.2.2 PIN Efflux Proteins

It was already in 1959 when the first *pin-formed 1* (*pin1*) mutant of *A. thaliana* was isolated, but during these days a function in response to the phytohormone giberellin was considered. More than 30 years later the first link between PIN proteins and polar auxin transport was published based on phenotypic observations of NPA (1-naphthylphthalamic acid) treated plants. The synthetic auxin efflux inhibitor phenocopies the pin-formed inflorescence in wild-type plants, a hallmark for defective auxin transport (Figure 8c). Additionally, analysis of transport activities showed a decreased IAA transport in *pin1* hypocotyls (Galweiler et al., 1998; Okada et al., 1991).

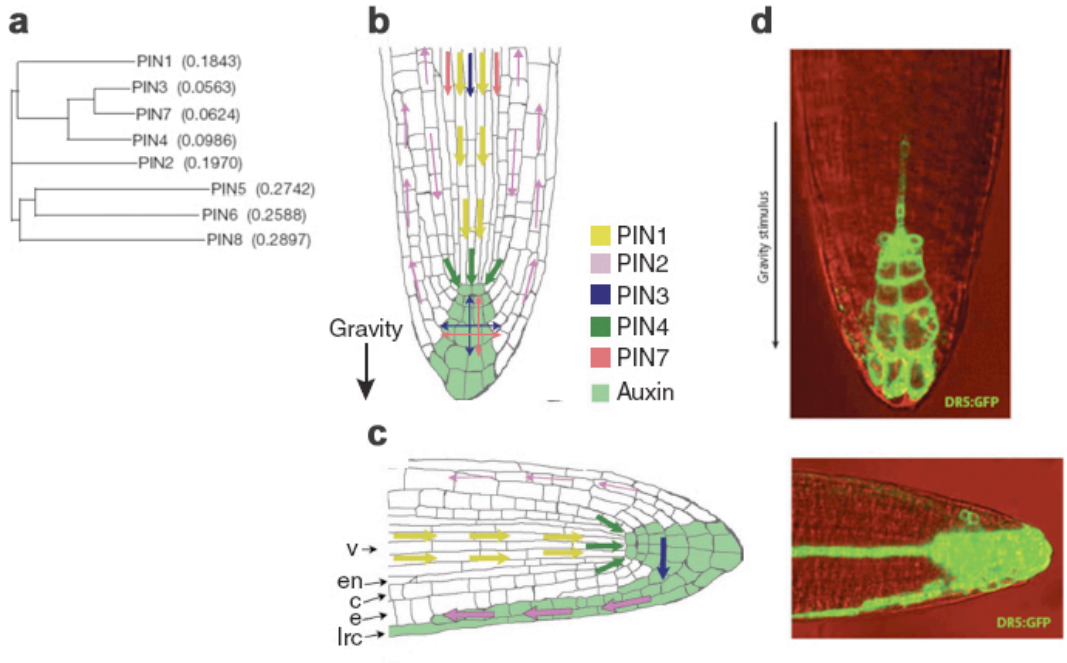


Figure 6. Phylogenetic tree of PIN proteins and their localization during root development

a) Phylogenetic tree of the Arabidopsis PIN protein family with indicated distances. PIN5, PIN6 and PIN8 represent the distant subclade. (From Mravec et al. 2009).
b and **c**) Primary root tip and gravitropism: auxin distribution (green) and polarity of the PIN localization and auxin fluxes (depicted by arrows) in the root tip (b) and effect of a gravistimulation on the auxin distribution and auxin fluxes (c). In the central vascular cylinder, PIN1 is acropetally (root-tip facing) localized and participates in the transport of auxin from the shoot to the root tip. PIN2 exhibits a basal and localization in root epidermal cells consistent with its apparent role in redirection and reflux of auxin at the root tip. PIN3 exhibits an apolar orientation in root columella cells, but relocalizes in the direction of auxin movement upon gravistimulation. PIN4 exhibits mixed polar and apolar localizations in the provascular quiescent centre and daughter cells and functions in root meristem patterning. PIN7 acts similar like PIN3.
d) Pattern of active auxin in the rot tip visualized with the auxin responsive element DR5::GFP.
v: vasculature cylinder, en: endodermis, c: cortex, e: epidermis, lrc: lateral root cap. (From Robert and Friml 2009).

Now after intensive years of research other single and multiple *pin* mutants were also identified. They show typical defects in auxin-related processes, such as tropisms (*pin2*), embryogenesis, root meristem patterning, organogenesis, and vascular tissue differentiation (Benkova et al., 2003; Blilou et al., 2005; Friml et al., 2002a; Friml et al., 2003; Friml et al., 2002b; Luschnig et al., 1998; Scarpella et al., 2006). Furthermore, PIN-mediated auxin transport seems to facilitate parasitic nematode infection in plants (Grunewald et al., 2009). Hence depending on the developmental stage and tissue PIN

proteins have different functions and subcellular distributions (Figure 6).

In *Arabidopsis* the PIN-FORMED (PIN) protein family consists of eight members with a predicted topology reminiscent of carrier proteins. All PIN proteins contain two hydrophobic domains, each with five trans-membrane helices, separated by a hydrophilic loop. New findings indicate that members of the PIN protein family separate into two distinct subclasses: the ancient ER-localized PIN5-type (PIN5, PIN6 and PIN8) and the plasma-membrane-localized PIN1-type proteins (PIN1, PIN2, PIN3, PIN4, PIN7). The latter show a direct role in the classical cell-to-cell transport, whereas PIN5 regulates the cellular homeostasis by mediating auxin compartmentalization from the cytosol into the ER lumen (Mravec et al., 2009). The common characteristic for PIN5-like proteins is the short hydrophilic loop. Although PIN6 and PIN8 show also ER localization their function is still unclear (Figure 6).

Importantly, relocalization of PIN1-type proteins in the plasma membrane correlates with a redistribution of auxin in a given tissue (Figure 6). Endosomal trafficking by clathrin-dependent endocytosis (Dhonukshe et al., 2007) or ARF GEF-dependent exocytosis of PIN1 (Geldner et al., 2001) enables a relocalization and thus a redirection of the auxin flow (Figure 12). Interestingly, after *de novo* synthesis PIN1 is first delivered to the plasma membrane in a non-polar manner and its polarity is established in a second step by subsequent endocytic recycling (Dhonukshe et al., 2008). The mechanism of PIN cycling is one out of few examples how auxin fluxes can be regulated and is therefore an intensively studied research field. For more information please refer to the following reviews: Feraru and Friml, 2008; Kleine-Vehn and Friml, 2008; Zazimalova et al., 2007.

One should not forget that although PINs account for the transport direction, auxin is its own organizer in determing its efflux rate and direction (Friml and Palme, 2002). Auxin influences transcription, turnover and membrane localization of PIN proteins. It interferes with the endocytosis of PIN proteins in order to increase the PIN abundance and activity on the plasma membrane (Paciorek et al., 2005). Another outstanding example of auxin-dependent reorganization of plant tissues is based on the **Canalization Hypothesis** proposed by Sachs (Sachs, 1986). Thus auxin can induce, by a positive feedback mechanism, the capacity and polarity of its own transport, leading to the formation of an auxin canal that final differentiates into a new vascular strand (Benjamins and Scheres, 2008). Additional consideration assumes PIN1 as the main mediator for vascular development. In leaves, mesophyll cells localize PIN1 along auxin fluxes, thus further enhancing auxin streams and cell polarization. However, it is still unclear how cells sense the rate and direction that auxin flows through them. It remains unlikely that putative auxin receptors like TIR1 (as it is localized in the nucleus) or ABP1 (as it binds auxin in the ER) do so. According to the fact that vein formation can be blocked by NPA, a synthetic auxin transport inhibitor that specifically binds to ABCB1, it is likely that a protein of the auxin efflux complex senses the directionality of the auxin flux (Hossel et al., 2005; Merks et al., 2007).

3.4.2.3 ABCB Transporter Family

According to the fact that auxin distribution is a complex phenomenon, it is not surprising that more than one mediator is involved in the auxin efflux system in plants. Although PIN proteins show a redundant function in mediating PAT (Vieten et al., 2005) and seem to form a well-buffered system to establish auxin directionality, phospho-glycoproteins (PGPs), referred to as ABCBs in the following, contribute significantly to the robust auxin distribution network (Figure 5 and 7). Moreover, depending on the developmental stage and cellular identity, auxin transport specificity and capacity is regulated by a coordinated interaction of PIN and ABCB transport proteins (Blakeslee et al., 2007; Mravec et al., 2008). The latter are our main research interest and belong to the B subgroup of the ancient adenosine triphosphate (ATP) -binding cassette (ABC) transporter superfamily, which are plant orthologues of the mammalian multi-drug-resistant (MDR) transporters.

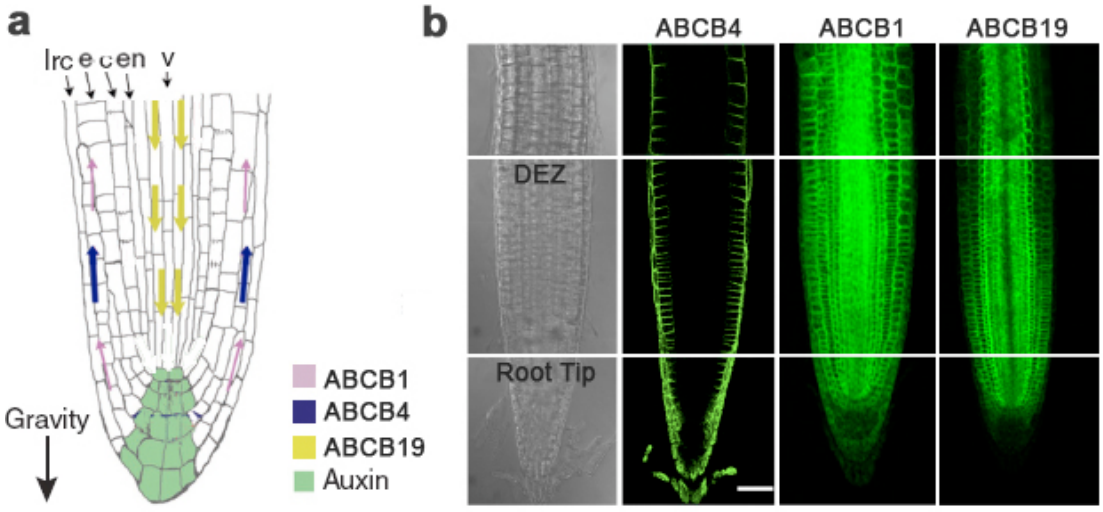


Figure 7. Localization of ABCB proteins the primary root

a) Primary root tip: auxin distribution (green) and direction of the ABCB-mediated transport fluxes (depicted by arrows) in the root tip. ABCB19 is expressed in the central cylinder and the cortex of the root apex, where auxin is transported acropetally and centripetally. These processes help balance expansion rates of cells on sides of the growing zone to guide vertical root growth. ABCB4, together with ABCB1, is an important contributor to basipetal auxin transport. It plays a role in controlling differential growth during gravitropism, perhaps by affecting the auxin asymmetry that drives the process. v: vasculature cylinder, en: endodermis, c: cortex, e: epidermis, lrc: lateral root cap.
b) Expression pattern of ABCB4, ABCB1 and ABCB19 in the primary root by confocal microscopy analysis. From left to right: bright field median sectional views of the root, DEZ: distal elongation zone; ABCB4::ABCB4:GFP (from Cho et. al, 2007); ABCB1::ABCB1:GFP and ABCB19::ABCB19:GFP (by S. Henrichs).

In Arabidopsis 29 proteins are members of the ABCB subgroup (Verrier et al., 2008) including 21 expressed, full-length P-glycoprotein transporters that show different functions and distinct or overlapping expression patterns throughout all stages of plant development (reviewed by (Titapiwatanakun et al., 2009). Until today, in Arabidopsis four members are characterized biochemically in more detail and, except ABCB14, which acts as a malate importer (Lee et al., 2007), they act as auxin transporters: ABCB1 (Geisler et al., 2005; Geisler and Murphy, 2006) and its closest homologue ABCB19 are auxin exporters while ABCB4 (Santelia et al., 2005; Terasaka et al., 2005) is

a conditional auxin efflux/uptake transporter (Titapiwatanakun et al., 2009; Yang and Murphy, 2009) (Figure 7).

As in other eukaryotic organisms, plant ABCB genes encode predicted proteins of about 1250 amino acids (125–140 kDa). ABCBs function as a "homodimer" (Ramaen et al., 2005). Each ABCB monomer consists of a transmembrane domain (TMD) composed of six hydrophobic α-helices embedded in the membrane bilayer, followed by a cytosolic nucleotide-binding domain (NBD), the site of ATP binding and hydrolysis. The two similar halves are intra-molecularly connected by a linker domain of about 60 amino acids, which characteristically shows a strong divergence between different ABCBs. Hence, in simple words ABCBs consist of four domains, two NBDs for the energy supply and two TMD involved in substrate recognition and translocation (Figure 10).

ABCB1/PGP1 was the first characterized member of the Arabidopsis ABCB subfamily, accidentally identified in a screen for herbicide resistance (Dudler and Hertig, 1992; Dudler and Sidler, 1998). Later it was shown that the resistance was only of little value and observed only in combination with the action of an apyrase, a calcium-activated plasma membrane-bound enzyme that catalyses the hydrolysis of ATP (Windsor et al., 2003). A connection between ABCB proteins and a function during hormone transport was suggested by Sidler et al., (1998). After phenotypic observation of cell elongation in ABCB1 overexpressing plants they conclude that a "conceivable function of AtABCB1 would therefore be the export of a hormone-like compound from the shoot apical region that would regulate hypocotyl cell length." However the first clear indications of an ABCB1 and ABCB19 function during polar auxin transport arose from the characterization of *abcb1/abcb19* Arabidopsis mutant plants that showed an impaired auxin transport activity in the hypocotyls and the inflorescence (Geisler et al., 2003; Lin and Wang, 2005; Noh et al., 2001). Additionally analysis of long-known mutations in crop plants that exhibit agronomic significance showed that mutations of ABCB1 orthologues in Maize (*brachytic2/br2*) and Sorghum bicolor (*dwarf3/dw3*) cause a compact plant growth due to a decrease of auxin transport (Multani et al., 2003). However, clear biochemical evidence for an ABCB1-mediated IAA transport derived from a publication in 2005 from Geisler et al. Heterologous expression of Arabidopsis ABCB1 in yeast (*S.cerevisae*) and mammalian cells results in an enhanced efflux of IAA and 1-NAA, which can be blocked by auxin transport- and ABC transporter inhibitors. A direct role for ABCB1 and ABCB19 in the auxin transport machinery was further confirmed when mesophyll protoplasts of either *abcb1* or *abcb19* mutant plants showed a decrease of natural and synthetic auxin efflux and a reduced sensitivity to auxin transport inhibitors. Moreover, the ABCB1 promoter contains an auxin responsible element (ARE) resulting in an auxin dependent ABCB1 expression that can be upregulated by an increased auxin concentration (Geisler et al., 2005; Geisler and Murphy, 2006).

ABCB19, the closest homologue of ABCB1, is the most highly expressed family member in Arabidopsis roots and hypocotyls (Birnbaum et al., 2003; Blakeslee et al., 2007; Noh et al., 2001). Auxin transport from the shoot apical meristem down into the root tip is reduced by almost 80% in *abcb19* hypocotyls (Blakeslee et al., 2007) and in primary roots (Lewis et al., 2007). Heterologous

expression of the AtABCB19 in HeLa cells leads to an enhanced auxin transport that can be blocked by the synthetic inhibitor NPA (Blakeslee et al., 2007; Bouchard et al., 2006). Moreover hetero-logous expression of ABCB19 is now possible in fission yeast (*S.pombe*) and leads to a strong IAA export (Yang and Murphy, 2009).

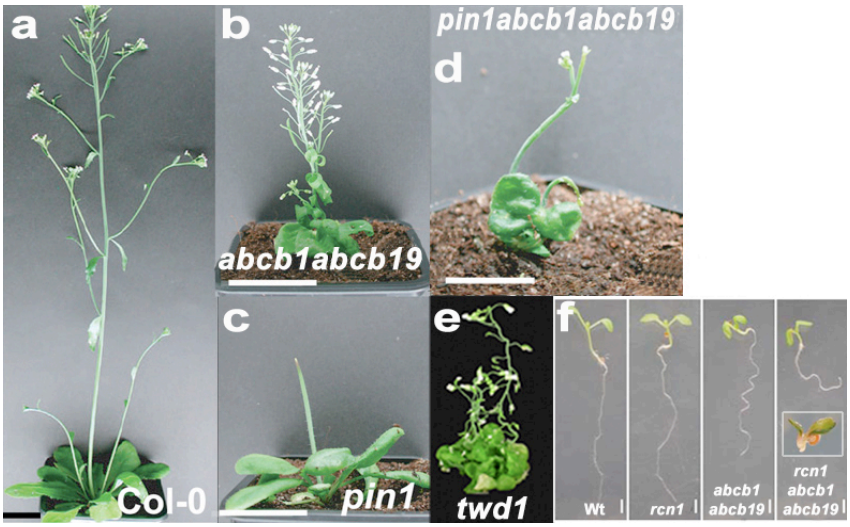


Figure 8: Auxin phenotypes in Arabidopsis mutant plants

a) Wildtype Columbia (Col-0) plant.
b) *abcb1abcb19* plant.
c) *pin1* plant.
d) *pin1abcb1abcb19* plant.
e) *twd1* plant.
f) Root phenotypes of the wildtype Ws, *rcn1*, *abcb1abcb19* and *rcn1abcb1abcb19*,
Inset: rootless defects in 11% of the *rcn1abcb1abcb19* seedlings (*n*=97).
Bar= 5cm in a) to e); f) Bar=2mm.
(Modified from Blakeslee et al., 2007, Mravec et. al., 2008)

ABCB1 and ABCB19 seemed to have partially overlapping functions to coordinate auxin export (Noh et al. 2001). Although no embryo patterning defect was reported in the *abcb1/abcb19* double mutant, ABCB1 and ABCB19 are expressed from the earliest embryonic stage on. Later in development, single knock-out mutants both exhibit a weak phenotype compared to the wild type, whereas the *abcb1abcb19* double-mutant causes dwarfed growth with short inflorescences, reduced axillary and secondary inflorescences, and reduced numbers of rosette and cauline leaves, which were smaller and curled; flowers and siliques were present (Figure 8b) (Blakeslee et al., 2007; Mravec et al., 2008).

ABCB1 is expressed in all root cells, except for the columella cells. Protein localization studies of ABCB1 proteins in primary roots of Arabidopsis seedlings showed an apolar localization in the plasma membrane of meristematic root cells, whereas a polar accumulation was detected in root epidermal cells in the elongation zone (Geisler et al., 2005) and also during embryo development (Mravec et al. 2008). ABCB19 gene expression is restricted to the endodermis and the pericycle. In the root, ABCB19 is mainly symmetrically localized in the vascular bundle sheet cells, the cortex and the endodermis (Figure 7b) (Wu et al., 2007).

In mammals, ABCBs are dynamically trafficked through early endosomes in an actin-dependent manner (Fu and Roufogalis, 2007; Orlowski et al., 2006). However, less is known about the regulation of ABCB subcellular localization in plants. The ABCB19-specific growth inhibitor gravacin has recently been shown to repress ABCB19 maturation and delivery to the plasma membrane from endomembrane sorting compartments in Arabidopsis. Experimental evidence suggests that plasma membrane lipid microdomains might play a role in regulating both PIN and ABCB membrane localization (Rojas-Pierce et al., 2007).

3.4.3 Root Gravitropism – an Example of Auxin in Action

Plant roots have the ability to alter their growth direction to maintain a set angle with the gravity vector (gravitropism). Root gravitropic bending is a hallmark of an auxin-mediated response and an excellent example of an environmental signal utilizing auxin to trigger plant orientation. Here, combined local auxin biosynthesis and polar transport leads to a highly specific auxin distribution in the root tip that is regulated by different proteins and endogenous compounds (Robert and Friml, 2009).

In a vertically grown root, auxin undergoes polar transport in two directions. There is transport from the root-shoot junction to the root apex ('**acropetal**') and a redirection towards the base ('**basipetal**') through the lateral root cap (Figure 4) (Baluska et al., 2005). A stable reflux system finally recycles newly synthesized and "imported" active auxins (Swarup et al., 2005) and ensures a definitive meristematic zone that is protected by a lateral root cap (Petersson et al., 2009). The acropetal auxin transport mobilizes shoot-derived auxin to the root apex through the stele, a transport route that is regulated by PIN1, PIN3, PIN4, PIN7 and ABCB19 (Lewis et al., 2007; Robert and Friml, 2009; Wu et al., 2007). In the columella cells PIN3 and PIN7 redirect auxin into the lateral root cap and epidermis. Here, AUX1 and PIN2 (Swarup et al., 2005) together with ABCB1 and ABCB4 (Santelia et al., 2005) further transport auxin up into the elongation zone. Finally, auxin recycles back into the vasculature, which is mediated by PIN1, PIN3 and PIN7 (Blilou et al., 2005) and probably ABCB19 (Figure 6 and 7) (Blakeslee et al., 2007).

Gravity is perceived in columella cells by the sedimentation of gravity-sensing organelles (statoliths) in the statocysts of the root tip (Figure 6c). This process is closely followed by relocation of PIN3 auxin transporters within these cells (Friml et al., 2002b), which in turn redirect the auxin flow to the adjacent lateral root cap and epidermal cells. Auxin is further transported by a combined action of AUX1-mediated in- and PIN2-mediated efflux to the elongation zone. Here auxin accumulation inhibits cell elongation and thus leads to downward bending of the root tip. However, after gravistimulation, proteasomal degradation of PIN2 at the upper side of the root results in asymmetric distribution of PIN2 (Abas et al., 2006; Kleine-Vehn et al., 2008). Additionally, ABCB1 and ABCB4 are also important contributors to basipetal auxin transport as they play a role in controlling differential growth during gravitropism, perhaps by affecting the auxin asymmetry that drives the final root bending (Lewis et al., 2007).

Auxin activity can be visualized indirectly *in vivo* by the auxin responsive **DR5rev::GFP** reporter construct roughly reflecting the levels of free, active auxin in the cell (Figure 6d). In wild type plants, a localized signal in the quiescent centre, the columella initials and the mature columella cells is visualized (Ottenschlager et al., 2003; Ulmasov et al., 1997). Indeed it was shown that the DR5 element is also activated by brassinosteroides, but errors in the auxin pattern by brassinosteroides can be widely excluded due to the fact that this cross-reaction occurs only in aerial parts of cotyledons (Nakamura et al., 2003).

3.5 Regulation of Auxin Transport

Polar auxin transport is regulated by many different cellular mechanisms - and specifically by the auxin molecule itself (Paciorek et al., 2005). Due to the chemiosmotic model efflux proteins determine the quantity and directionality of the auxin transport and as a consequence any kind of efflux protein regulation finally affects auxin transport. This can happen on different cellular levels:

1. Protein abundance is influenced by transcriptional regulation (Geisler et al., 2005; Noh et al., 2001; Terasaka et al., 2005; Vieten et al., 2005) and therefore by auxin itself (Paciorek et al., 2005) or endogenous flavonoids (Peer et al., 2004; Santelia et al., 2008), translation, protein degradation (Kleine-Vehn et al., 2008; Laxmi et al., 2008; Malenica et al., 2007) and hormonal cross-talk (Dello Ioio et al., 2008; Pernisova et al., 2009).

2. **Protein localization** is achieved by subcellular trafficking mechanism (Dhonukshe et al., 2007; Geldner et al., 2001), by membrane composition (Titapiwatanakun et al., 2009) and protein phosphorylation events (Michniewicz et. al. 2007).

3. **Protein transport activities** are regulated by endogenous flavonoids (Bouchard et al., 2006), protein-protein interaction (Bailly et al., 2008; Blakeslee et al., 2007; Michniewicz et al., 2007) and probably by protein phosphorylation-dependent regulation.

In the next chapter some of the listed regulatory mechanisms will be discussed in more detail.

3.5.1 Transcriptional Regulation of Auxin Transport

3.5.1.1 Transcriptional Regulation by Auxin

Although auxin transport is regulated by many different cellular mechanisms it basically triggers it own distribution by a transcriptional feedback loop that involves the binding of auxin to the TIR1 receptor (Transport Inhibitor Response1), thereby regulating the transcriptional activity of ARF proteins via the proteolysis rate of Aux/IAA proteins (see also chapter 3.3, Figure 3). During this robust feedback loop, the expression of Aux/IAA genes is induced by auxin, which in turns lower the sensitivity of cells towards auxin, while ARF proteins, which regulate the auxin-responsive gene transcription, are repressed by the Aux/IAA interaction. The self-regulatory mechanism further allows the auxin-dependent degradation of Aux/IAA proteins that releases ARF proteins to transcribe auxin-regulated genes, including the *Aux/IAA* genes (Benjamins and Scheres, 2008). Here the combination of auxin-mediated expression of *Aux/IAA* genes and the auxin-dependent degradation of the Aux/IAA proteins balances the auxin response and auxin transport. High auxin concentrations are sensed by TIR1, which results in the breakdown of Aux/IAAs and the subsequent release of ARF transcription factors that upregulate the expression of auxin transport components, which transport auxin out of the cell. And although this auxin signalling pathway is relatively short, the number of Aux/IAA (29) and ARF (23) genes is high, providing plants with many possible combinations for auxin-regulated transcription (Quint and Gray, 2006).

However, gene array experiments demonstrate that hundreds of genes change their expression in response to auxin (Pufky et al., 2003) and analysis of the promoter regions of these gene led to the identification of auxin response elements (AREs) (Hagen and Guilfoyle, 2002). Also the transcription of mainly all known proteins of the auxin transport machinery (ABCBs, PINs, PID, AUX1/LAX) is influenced by auxin, probably via signaling through the TIR1 cascade. The transcriptional regulation of PIN proteins was analyzed in more detail and it has been shown that functional redundancy among the members of the PIN family involves cross-regulation of the expression of their genes (Vieten et al., 2005). This suggests a feedback regulation, whereby deviations in the auxin distribution induce ectopic gene expression of PINs, and therefore a correction of the auxin distribution pattern. In the root tip this feedback is mediated by two closely related members of the **PLETHORA** family of transcription factors (Blilou et al., 2005). Further, the regulation of PIN gene transcription by the TIR1 network has also been observed in various contexts such as the shoot apical meristem and the embryo.

3.5.1.2 Transcriptional Regulation by Flavonoids

Flavonoids are polyphenolic endogenous secondary metabolites found in all vascular and non-vascular plants. They are considered multifunctional autocrine effectors that mediate plant growth and development, which have species-specific roles in nodulation, fertility, defense and UV protection (Peer and Murphy, 2007). Further, flavonoids have been shown to modulate transport of auxin in addition to auxin-dependent tropic responses. However, flavonoids are not essential regulators of these processes because transport and tropic responses occur in their absence. It is known that flavonoids interact with ABCB proteins that as a consequence inhibit their transport activity (Bailly et al., 2008; Bouchard et al., 2006). However, a regulatory function of flavonoids on gene transcription remains less clear. Some indications suggest that the transcriptional effects of flavonoids on members of the PIN protein family in expression and subcellular protein localization could be attributed indirectly to altered local auxin concentrations, rather than to a direct flavonoid interaction. This is based on observations made in the *transparent testa* (*tt*) Arabidopsis mutants showing an impaired flavonol biosynthesis. Here, changes in PIN gene expression and protein localization could be mimicked by exogenous application of auxin or ATIs. However, opposite regulation of PIN1 and PIN2 by flavonoids was reported: PIN1 is delocalized from the plasma membrane and gene expression is depressed in the absence of flavonoids, whereas localization of PIN2 is unaffected and gene expression is enhanced (Peer et al., 2004; Peer et al., 2001; Peer and Murphy, 2007). Heterologous system transport assays with PIN proteins in the presence of flavonoids show no effect on the transport activity compared to control conditions (Blakeslee et al., 2007; Bouchard et al., 2006). Finally, results from my first project of my PhD studies indicate that exogenously application of quercetin to Arabidopsis seedlings induces the ectopic expression of PIN1 in a *pin2* loss-of-function background. The well-known redundancy of PIN proteins is therefore linked to the transcriptional regulation by flavonoids (Santelia et al., 2008).

3.5.2 Post-Transcriptional Regulation of Auxin Transport

Auxin transport is regulated by many different post-transcriptional modifications. In the next chapter a few examples of regulatory mechanisms, that are of interest for my project are discussed in more detail.

3.5.2.1 Regulation by Protein-Protein Interaction

3.5.2.2 ABCB- and PIN protein interaction

ABCBs are ancient auxin efflux proteins and are found from unicellular algae to angiosperms, whereas PINs occur much later in evolution in multicellular land plants (Figure 13) (Galvan-Ampudia and Offringa, 2007). The evolutionary history reflects that ABCB and PIN proteins might work independently, nevertheless, several lines of evidence show that they also interact directly or indirectly in order to coordinate auxin transport (Bandyopadhyay et al., 2007; Blakeslee et al., 2007; Mravec et al., 2008). A mutual effect of the proteins is based on observations in HeLa and/or yeast cells, when heterologous co-expression of ABCB1 and ABCB19 in the presence of PIN1 results in enhanced auxin efflux and an increase of substrate specificity. Cellular localization analysis of PIN and ABCB proteins in Arabidopsis shows a more complex picture where ABCB1/ABCB19 co-localize with different PINs depending on the tissue, developmental stage or cellular identity. For instance, PIN2 co-localizes with ABCB1 in root epidermal cells. Co-immunoprecipitation and yeast two-hybrid screens further underlined the physical interaction of these proteins where the C-terminal domain of ABCB19 interacts with PIN1 (Blakeslee et al., 2007) and ABCB19 stabilizes plasma membrane microdomains that enhances PIN1 auxin transport activity (Titapiwatanakun et al., 2009). Additionally, phenotypic analysis of the *pin1 abcb1 abcb19* mutant compared to *abcb1 abcb19* and *pin1* suggest a synergistic genetic interaction between PIN1 and the ABCB proteins (Figure 8a-d). Furthermore, phenotypic observations during development indicate that ABCB- and PIN1- mediated transport systems are required for the formation of well-defined auxin maxima during embryogenesis (Mravec et al., 2008). However, for PIN1 and ABCB1 protein interaction, the case is less clear and evidence for protein-protein interaction in the yeast two-hybrid system are missing which is probably due to an indirect interaction[1]. On the other hand, *pin2 abcb1 abcb19* phenotypes also show a synergistic agravitropic phenotype, which makes one speculate on a possible PIN2-ABCB interaction (Blakeslee et al., 2007). After all, phenotypes of *pin* and *abcb* mutants suggest discrete, sometimes complementary roles in auxin transport mechanisms that cooperate to mediate embryogenesis, organogenesis and root gravitropism.

In summary, it seems that ABCBs primarily function in the maintenance of long-distance polar transport and movement of auxin out of apical tissues, whereas PIN proteins are more important for a local redirection (Figure 6 and 7). It is important to point out that ABCB and PIN proteins can act independently, additively or synergistically and this further depends on the cellular identity and

[1] This is supported by the fact that based on unpublished data from our lab, PIN1 seems to interact *in planta* with immunophilin-like ABCB1 regulator, TWISTED DWARF1.

developmental stage. In the meristematic tissue, where cells are smaller and auxin concentrations are higher, ABCBs might act independently of the PIN proteins, in order to mediate apolar auxin efflux and control the amount of auxin available in the cells for the PIN-mediated vectorial transport. In addition, at the polar domains of mature cells where the ABCB and PIN proteins co-localize and interact, ABCBs might act synergistically with PIN proteins to enhance efflux activity, and probably by regulating PIN stability at plasma membrane microdomains (Bandyopadhyay et al., 2007; Geisler and Murphy, 2006).

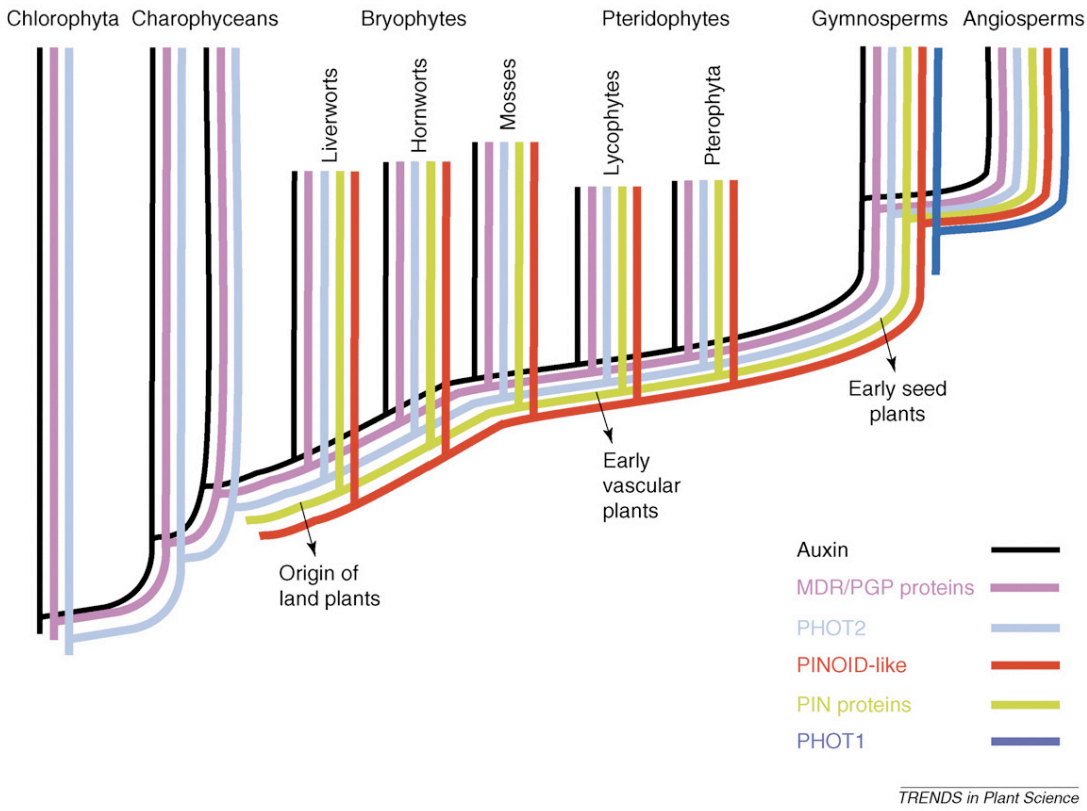


Figure 9: Evolution of the auxin transport machinery

Phylogenic tree of the plant kingdom, depicting the presence of genes involved in auxin biosynthesis, or genes encoding phototropins, AGC3 (PINOID-like) kinases, ABCB transporters and PIN proteins. Auxin and auxin-biosynthesis genes have been identified in a range of green plants, from unicellular algae to angiosperms, as have genes encoding PHOT2-like proteins and ABCB transporter proteins. By contrast, genes encoding the AGC3 kinases and PIN auxin efflux carriers occur only later in evolution in land plants. Therefore, the PHOT2-like proteins probably represent the most ancient AGCVIII protein kinases from which the other AGCVIII kinases evolved. (From Galvan-Ampudia and Offringa 2007).

3.5.2.3 ABCB1- and TWISTED DWARF1 protein interaction

Although two classes of auxin transporters/carriers and their interaction potential have partially been discovered, there is still a lack of understanding how auxin gradient formation is regulated in detail. The developmental changes that are mediated by different combinations of PIN-ABCB interaction partners do not explain the variety of effects they cause. Another important puzzle piece in the auxin transport network is the immunophilin-like FKBP42 TWISTED DWARF1 (TWD1), that is involved in plant development by modulation of ABCB1 and ABCB19-mediated auxin transport (Bailly et al., 2008; Bouchard et al., 2006; Geisler and Bailly, 2007).

Loss-of-function of *twd1* (*ucu2*) causes a pleiotropic auxin-related phenotype with a disoriented growth that leads to twisted organs and a drastic reduction of cell elongation that terms them “dwarfed” (Figure 8e) (Geisler et al., 2003). Both *twd1* and *abcb1 abcb19* mutants show similar defects, like reduced plant growth, reduced auxin transport rates and elevated levels of free auxin, whereas morphological similarities are more obvious at early developmental stages. Interestingly *TWD1* gain-of-function had no effect on the net IAA transport compared to the wild type indicating a regulatory role during auxin transport (Bouchard et al., 2006; Perez-Perez et al., 2004).

TWD1 belongs to the FK506-binding protein (FKBP)-type family of *cis-trans*-peptidyl-prolyl isomerases (PPIases). The TWD1 characteristic C-terminus forms an in-plane membrane anchor that localizes the protein to the membrane (Geisler et al., 2004; Kamphausen et al., 2002; Scheidt et al., 2007). The tetratricopeptide repeat (TPR) domain shows protein-protein interaction capabilities and interacts directly with the vacuolar multidrug-resistant associated ABCC transporters (MRP1, MRP2) and AtHsp90 (Geisler and Bailly, 2007; Kamphausen et al., 2002). In respect to auxin research, the most direct functional interaction takes place between the N-terminal PPIase/FK506-binding domain (FKBD) of TWD1 with the C-terminal nucleotide binding domains (NBDs) of ABCB1 and ABCB19 in order to act as a mediator of ABCB1- and ABCB19-regulated auxin efflux (Figure 10) (Bailly et al., 2008; Bouchard et al., 2006; Martinoia et al., 2002). This protein-protein interaction takes place on the plasma membrane and modulates movements of auxin out of apical regions and into long-range auxin transport routes (Lewis et al., 2007). When co-expressed with ABCB1, TWISTED DWARF1 showed opposite effects on ABCB1-mediated auxin efflux activity in yeast, HeLa and plant cells: inhibition was observed in yeast, whereas activation was seen in HeLa and plant cells (Bouchard et al., 2006). This suggests that the same interaction partner modulates ABCBs in a reverse manner depending on the expression system that most likely reflects the presence of an organism-specific additional modulator. Auxin transport inhibitors and endogenous flavonoids, inhibitors of mammalian and plant ABCBs, disrupt the ABCB1-TWD1 interaction (Bailly, Sovero et al. 2008). And, taking into account that TWD1 co-localizes with ABCB1 and ABCB19 (unpublished) and is expressed with a low abundance, it supports the fact that TWD triggers a transient regulatory function during plant development.

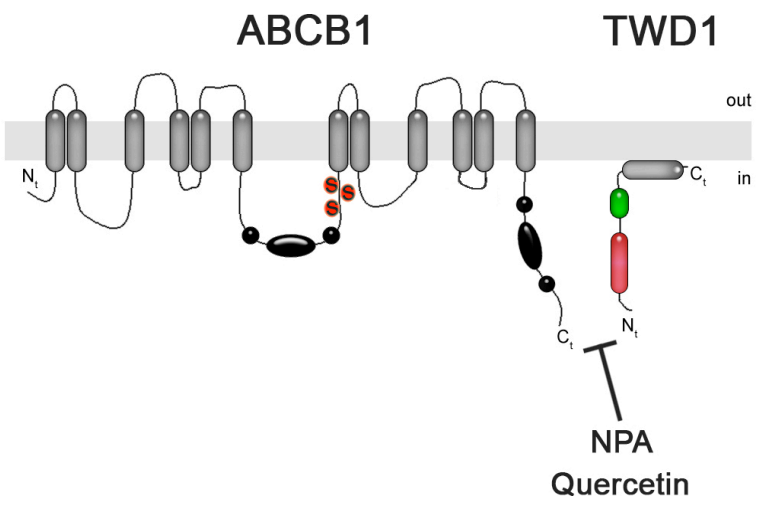


Figure 10: Protein-Protein Interaction of ABCB1-TWD1

The C-terminal domain of ABCB1 interacts with the N-terminal PPIase/FK506-binding domain (red) of TWD1, an interaction that can be destabilized in the presence of NPA or quercetin. The tetratricopeptide repeat (TPR) domain is shown in green. Red dots in ABCB1 represent an accumulation of phosphorable serine residues in the linker domain (modified from Bailly et al., 2008).

Finally, it is also likely that TWD1 has a function during **brassinosteroid** signaling or the correlated cross talk with auxin. *Twd1* loss-of-function is insensitive to exogenous application of brassinosteroids and resembles the ***bri1*** phenotype (BRASSINOSTEROID-INSENSITIVE1), a leucine rich receptor like kinase (Kim et al., 2007b; Nemhauser et al., 2004; Perez-Perez et al., 2004). In addition, both proteins are located to the same membrane (Friedrichsen et al., 2000). Phosphorylation and cross-linking experiments with TWD1 and BRI1 might provide more evidence for this assumption.

3.5.2.4 Endogenous Auxin Transport Modulators - The Flavonoids

The secondary plant metabolites, flavonoids, are most probably auxin transport inhibitors. Quercetin, kaempferol and other aglycone molecules have been demonstrated to modulate the ABCB-mediated auxin transport directly (Bailly et al., 2008; Blakeslee et al., 2007; Bouchard et al., 2006; Geisler et al., 2005) and indirectly (Bailly, Sovero et al. 2008) and as a consequence enhance local auxin accumulation *in planta* (Peer et al., 2004). Flavonoids accumulate in tissues that transport auxin and accumulation increases in gravity-stimulated root tips. However flavonoids may act as non-essential regulators of these processes because transport and tropic responses, although delayed, occur in their absence (Brown et al., 2001; Peer and Murphy, 2007; Winkel-Shirley, 2002). Based on this, flavonoids are suggested to modulate the rate and extent of the gravitropic responses in the roots (Buer and Muday, 2004; Buer et al., 2007).

Initially the regulatory impact of flavonoids, specifically quercetin, was based on their ability to displace the synthetic auxin transport inhibitor NPA from plasma membrane and microsomal binding sites (Figure 5 and 10). The NPA-flavonoid interaction site was shown to be associated with ABCBs, whereas a weaker interaction site was associated with TWD1 and the membrane-anchored M1 aminopeptidase (APM1) (Bernasconi et al., 1996; Murphy et al., 2000; Peer and Murphy, 2007). Detailed analysis reveals that application of flavonoids lead to destabilization of the ABCB-TWD1 protein complex indicating a regulatory function during auxin transport by affecting protein-protein interaction (Bailly et al., 2008).

As a final note, flavonoids show evolutionary conserved functions and are also active in animals, where they are well known for their health-promoting effects to act as antioxidants and anti-carcinogenics (Rice-Evans, 2001). Specially kaempferol and quercetin inhibit human ABCB expression and reduce ABCB-mediated drug efflux in cancer cells resulting in a decreased multi-drug-resistance (Ferte et al., 1999; Limtrakul et al., 2005). Since no toxic effects have been reported for flavonoids they are of special interest as non-toxic chemo-sensing agents and studies on the molecular mechanism underlying the flavonol-effects are notably beneficial for cancer treatment.

3.5.2.5 Synthetic Auxin Transport Inhibitors

Synthetic auxin transport inhibitors (ATIs) have become important tools to study mechanisms of the polar auxin transport machinery. In general, exogenous applied ATIs interfere with the directional transport and detailed studies of their site of action reveal new insights into the biochemistry and physiology of polar auxin transport (Figure 11).

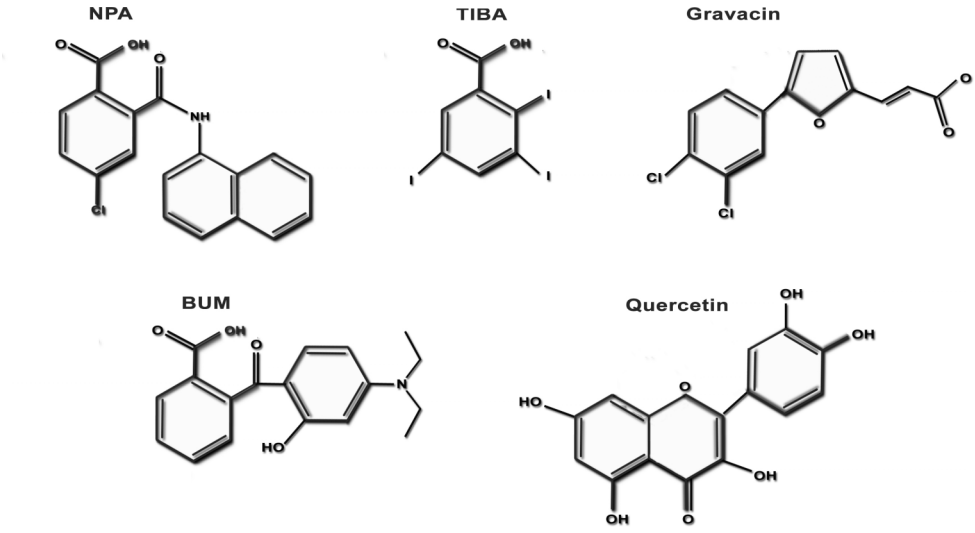


Figure 11. Auxin Transport Inhibitors

NPA (N-1-naphylphtalamic acid) is a well-known non-competitive auxin efflux inhibitor and herbicide (naptalam) (Figure 11). Although application of NPA to Arabidopsis wild type plants induces a *pin1*-like phenotype (Figure 8d), it specifically inhibits the ABCB1/19 mediated auxin efflux activity (Bailly et al., 2008; Bernasconi et al., 1996; Geisler et al., 2005; Noh et al., 2001). This idea is supported by the fact that ABCB1 and TWD1 have been identified as high- and low affinity NPA binding proteins (NBP) (Bailly et al. 2008). As a consequence, NPA has a destabilizing effect on the ABCB1-TWD1 protein interaction (Figure 10).

In plants, NPA interferes with the gravitropic response (Katekar and Geissler, 1975) and as a consequence auxin fluxes and gravitropism are less effected by NPA treatment in *twd1* and *abcb1/19*. Although in the heterologous system NPA inhibits ABCB1 and PIN1-mediated transport only slightly, the combined transport of ABCB1-PIN1 is blocked by nearly 90% (Blakeslee et al., 2007). Taking this into account, a reasonable scenario is that PINs function together with the ABCB-TWD1 complex to create an efficient NPA-sensitive auxin efflux mechanism. There are considerable parallels in the animal literature for ABC transporters acting as regulators of passive channels with which they interact physically (Vanoye et al., 1997).

Another, but only partially understood potential candidante as part of the NPA binding complex is the calossin-like TIR3/BIG, originally isolated in a mutant screen fo their resistance to auxin inhibitors

(Ruegger et al., 1997). *tir3* (*transport inhibitor response 3*) mutant plants show defects in auxin signaling and an auxin related phenotype. The functional TIR3/BIG protein contains several putative Zn-finger domains and might be involved in an unknown mechanism of vescile PIN1 trafficking (Paciorek et al., 2005, Sauer et al., 2006).

In contrast to NPA, **TIBA** (2,3,5-triiodobenzoic acid) employs different loci and mode of action and inhibits auxin efflux by directly competing with IAA at the efflux site (Figure 11) (Lomax et al., 1995). Importantly, TIBA had no significant effect on the TWD1-ABCB1 complex stability and auxin transport activity (Bailly et al., 2008). Furthermore, TIBA disrupts PIN vesicle trafficking processes by interfering with actin stability (Dhonukshe et al., 2008).

Another just recently identified ATI is **Gravacin** (gravitropism and vacuolar transport inhibitor) (Figure 11). It is a potent inhibitor of root and shoot gravitropism, auxin responsiveness and protein trafficking to the tonoplast in Arabidopsis. The major binding target is ABCB19 resulting in inhibition of gravitropic responses and auxin transport activity. Although Gravacin binding is also reduced in *twd1*, the gravacin effect on ABCB19 seemed to be indirectly linked to TWD1. Interestingly, the ABCB19-bound Gravacin can be displaced by NPA. Furthermore, E1174 was identified as an important residue for ABCB19 activity as it is involved in the interaction with PIN1. Because of its specific inhibitory effect on ABCB19 gravacin is a useful tool to dissect the role of ABCB19 without the inhibition of other auxin transporters, like PIN proteins (Rojas-Pierce et al., 2007).

The second part of my work characterizes a novel auxin transport inhibitor **BUM** that was identified by a chemical genomics approach (Figure 11). BUM is similar to NPA, but is active at much lower concentrations and acts predominantally on ABCB1 (Kim and Henrichs et al. 2009, resubmission).

3.5.2.6 Regulation of Auxin Transport by Protein Phosphorylation Events

Protein phosphorylation is a post-translational modification that is controlled by protein kinases and protein phosphatases, respectively. Reversible phosphorylation events influences protein function relatively fast and affects mainly every basic cellular process, including metabolism, growth, division, differentiation, organelle trafficking and membrane transport.

3.5.2.7 Regulation of PIN Localization by Protein Phosphorylation

Since a long time its is known that phosphorylation is a key regulatory mechanism for polar auxin transport (Delbarre et al., 1998), but detailed modifications are still unclear. Today, the best-characterized phosphorylation modification of PAT is the phosphorylation-dependent localization of PIN proteins, a process that is antagonistically regulated by the serine-threonine kinase **PINOID** (PID) and the trimeric serine-threonine protein phosphatase 2A (**PP2A**) (Figure 12). In particular *in vitro* and *in vivo* phosphorylation studies suggest that PID and PP2A antagonistically label PIN cargoes for trafficking to the appropriate membrane by phosphorylating the hydrophilic loop of PIN proteins (Michniewicz et al., 2007).

In mutant plants, *PID* loss-of-function or *35S::PID* gain-of-function changes the apical (shoot-tip-facing) or basal (root-tip-facing) cellular localization of PIN proteins influencing the direction of the auxin movement (Friml et al., 2004). As a consequence, in the *pid* mutant PIN1 localizes to the basal membrane of epidermal cells, which in turn redirects auxin away from the meristem and prevents the initiation of new lateral organs, thus resulting in a pin-shaped-like inflorescence with only a few flowers (Okada et al., 1991, Bennett et al., 1995, Christensen et al., 2000). This regulatory function seems to be conserved and also found in monocts, as the PID orthologue ZmBIF2 (BARREN INFLORESCENCE2) phosphorylates and mediates ZmPIN1 localization (Skirpan et al., 2009) and *ZmBIF2/OsPID* loss-of-function leads to defects in the development of the axillary meristem in maize and rice (McSteen et al., 2007, Morita et al., 2007). In the root tip of Arabidopsis seedlings, the *pid* mutation causes endomembrane accumulation of PIN2 without a shift in PIN2 polarity (Sukumar et al., 2009). On the other hand PID over-expression leads to basal-to-apical switch of PIN1, PIN2 and PIN4 in root cortex and lateral root cap cells and finally to a collapse of the root meristem due to auxin depletion (Michniewicz et al., 2007). Additionally, PID is an auxin responsive gene and as a consequence part of the auxin feedback loop (Benjamins et al., 2001). In summary this indicates that PID is a key modulator in the regulation of PAT to orient plant development.

PID belongs to the **AGC** family of serine/threonine kinases, which is named after the protein kinase A (PKA), cyclic GMP-dependent protein kinase (PKG) and protein kinase C (PKC) that are involved in receptor mediated growth factor signal transduction in animals. In plants, an orthologue subfamily of the AGC kinases, AGCVIII, has been identified. In Arabidopsis, this family comprises PID, which groups to the AGC3 clade together with AGC3-4, WAG1 and WAG2 (Galvan-Ampudia and Offringa, 2007). Loss-of-function mutations of *wag1 wag2* show an auxin-dependent root waving phenotype

(Santner and Watson, 2006) and here root curling is more resistant to NPA. Enhanced NPA sensitivity, the fact that the WAG kinases (like PID), are plasma membrane-associated and the enhanced expression in the root tips suggest that PID and WAG kinases act in the same or in a parallel pathway to regulate the auxin transport machinery (Santner and Watson, 2006). The ancient phototropins PHOT1 and PHOT2, which belong to the AGC4 group, are blue light receptors that trigger phototropic growth (Figure 9).

PID kinase activity is regulated by phosphorylation of the catalytic activation loop by 3-phosphoinositide-dependent kinase 1 (**PDK1**), which is an evolutionarily conserved mechanism for the regulation of AGC kinase activity (Zegzouti et al., 2006a; Zegzouti et al., 2006b). PDK1 recruits the kinases by binding to their hydrophobic C-terminal PDK1-Interacting-Fragment (PIF) domain. Interestingly, the majority of the Arabidopsis AGCVIII kinases, including PID, but excluding WAG1, WAG2, PHOT1, or PHOT2, have a PIF domain and are *in vitro* substrates of PDK1 (Bogre et al., 2003). However, in plants the significance the PDK1- dependent phosphorylation is still unclear, because PID is an auto-activating kinase, and it may therefore not need PDK1 activation during standard developmental programs.

Furthermore, PID activity is positively or negatively regulated by two Ca^{2+}-binding-proteins TOUCH3 (**TCH3**) or PINOID-BINDING PROTEIN1 (**PBP1**) which bind PID in a calcium-dependent manner thus regulating its kinase activity (Figure 12) (Benjamins et al., 2003). TCH3 is a calmodulin-related protein, PBP1 is a small protein with a single EF-hand and calcium influx and calmodulin inhibitors were found to enhance the activity of PID *in vivo* (Benjamins et al., 2003). Recent results indicate that this calcium-dependent regulation by TCH3 and PBP1 is conserved for all four AGC3 kinases (Galvan-Ampudia and Offringa, 2007).

New indications suggest that Broad Complex, Tramtrack, Bric-a-Brac (BTB) scaffold proteins are involved in the PID signaling pathway. In general scaffold proteins are characterized by the presence of multiple protein–protein interaction domains and are important for assembling transcription and signaling complexes. Characterization of one BTB protein, namely MACCHI-BOU4/ENHANCER OF PINOID/NAKED PINS IN YUC MUTANTS1 (MAB4/ENP/NPY1) of the NPH3-like family suggests a role during auxin transport (Cheng et al., 2007b; Furutani et al., 2007).

Moreover, a recent publication shows regulation of PID expression by the transcription factor INDEHISCENT (IND) during fruit development (Sorefan et al., 2009).

Finally, and in order to make the story complete, dephosphorylation of PIN proteins by the trimeric serine-threonine protein phosphatase 2A (**PP2A**) leads to a top-to-bottom switch in the cellular localization (Figure 12) (Michniewicz et al., 2007). In Arabidopsis there are three closely related regulatory A subunits of the PP2A - PP2AA1, PP2AA2 and PP2AA3. Preferentially one of the A regulatory subunits PP2AA1, called *ROOTS CURL IN NPA1* (*RCN1*), causes various developmental defects, including root agravitropism, cotyledon defects and root meristem collapse (Deruere et al., 1999; Garbers et al., 1996). *rcn1* was isolated in a screen for alterations in differential root elongation in the presence of NPA. It was shown that PP2A is a negative regulator of basipetal transport in the

root and as a consequence *rcn1* roots exhibit a significant delay in gravitropism, consistent with an increased basipetal auxin transport (Rashotte et al., 2001; Sukumar et al., 2009). Importantly, the *rcn1* gravitropic phenotype can be rescued by low concentrations of NPA, a concentration that is sufficient to block gravitropism in wild-type seedlings (Muday and DeLong, 2001). On the other hand the acropetal auxin transport is unaffected in *rcn1*, but shows a dramatic loss of NPA inhibition. Interestingly, *rcn1 pin2* double-mutant analyses indicate that elevated basipetal transport in *rcn1* does not require PIN2, leading to the suggestion that a NPA-binding protein is involved in this process (Figure 8f) (Rashotte, DeLong et al. 2001).

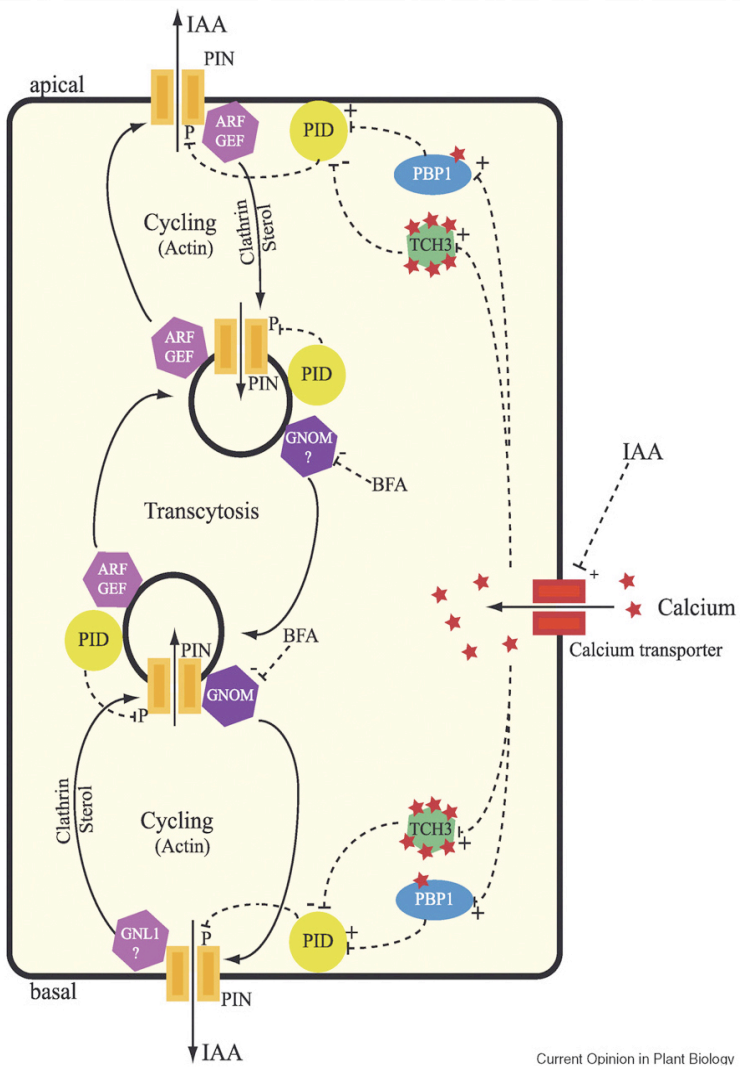


Figure 12: The PINOID kinase as a key regulator of the polar localization of PIN proteins

For a rapid regulation of PIN localization, they cycle between endosomal membrane compartments and the plasma membrane, via exocytosis and clathrin- and sterol-dependent endocytosis. This trafficking requires the action of ARF-GEF proteins. Movement of PIN-cargoes between the basal and apical cycling pathways is occurring via transcytosis. The PINOID (PID) labels PIN cargoes for trafficking to the apical plasma membrane by phosphorylating the hydrophilic loop of PIN proteins. PID activity is negatively or positively regulated through its calcium-dependent interaction with the calcium-binding proteins TOUCH3 (TCH3) or PINOID-BINDING PROTEIN1 (PBP1), respectively. Elevated auxin concentrations are well known to increase cytosolic calcium and, apart from the positive effect of auxin on PID expression, this could provide a second feedback loop by which auxin directs its own transport by regulating the subcellular localization of PIN proteins. (from Robert et al., 2008)

The impact of PID on the gravitropic response was addressed in a recent publication. Mutant analysis and pharmacological treatment with the kinase/phosphatase inhibitor staurosporine/cantharidin revealed that PID and PP2A antagonistically regulate basipetal auxin transport and gravitropic response in the root tip (Sukumar and Edwards et al. 2009). Loss of PID activity alters the PIN2-

mediated basipetal auxin transport and impedes the gravitropic response, without causing an obvious change in PIN2 cellular polarity. This finding indicates that PID promotes and enhances root gravitropism, but is not absolutely required. Furthermore, PID appears to have a specific regulatory effect on the basipetal transport machinery in the root, since the acropetal transport is unaffected in *pid*. This goes in hand with localization studies, here PID:YFP shows a polar localization in the epidermal and cortical cell layer that partially overlaps with PIN2 (Galvan-Ampudia and Offringa, 2007), whereas in the stele and endodermis PID is not expressed.

All these data are consistent with the concept of PID as a positive regulator of IAA efflux (Lee and Cho, 2006). However the direct affect of PID on the individual transporter activity have not yet been reported and will be addressed in the third part of my work.

3.5.2.8 Regulation of ABCB1 Activity by Protein Phosphorylation

Although phosphorylation events have an important effect on trafficking and polarity of PIN protein, ABCBs are also well-known targets of reversible phosphorylation-dependent regulation. Former studies from different organisms (human, mouse, hamster, protozoan *L. tropica*, yeast *S. cerevisiae*) reveal that ABCB1/MDR1/P-glycoprotein is phosphorylated in a so-called linker region that modifies the transport and the associated ATPase activity (Figure 10) (Idriss et al., 2000; Szabo et al., 1997). The linker region of about 60 amino acids represents the major target for ABCB1-phosphorylation (Chambers et al., 1993; Germann, 1996; Germann et al., 1996) and an accumulation of phosphorylatable serine residues were identified to be phosphorylated by protein kinase A and/or C (PKA/C), orthologues of the plant kinase PINOID (Chambers et al., 1990; Conseil et al., 2001; Orr et al., 1993). Interestingly, within the linker there are eight consensus phosphorylation sites for PKA and PKC (Glavy et al., 1997). However, many attempts have been made to correlate the degree of phosphorylation of ABCB1 with its drug efflux activity, but conflicting results emerged from various studies and were difficult to interpret. In summary, manipulations by site-directed mutagenesis of all serines in the linker region mimic permanent de/phosphorylation, however, none of these mutations caused a measurable change in drug resistance, drug accumulation, or rates of drug transport by ABCB1 (Germann, 1996; Goodfellow et al., 1996). Further indications for the regulatory function of the linker domain came from a study where a linker deletion construct of ABCB1 showed normal cellular localization but no drug-stimulated ATPase activity (Hrycyna et al., 1998). In general, most of the studies suffer from limitation of mutating a multiple of serines at once, rather than examining the effect of altering a single residue at a time. Multi-residue mutation may mask variations in the activity of ABCB1 resulting from a single phosphorylation. Indicative for this is the fact that certain serine residues are exclusively phosphorylated by PKC (S661) or PKA (S683).

Nevertheless, it was shown that phosphorylation modulates ABCB ATPase activity that correlates (Senior et al., 1995) and as a consequence regulates the drug transport properties (Szabo et al., 1997). Moreover, PKC positively regulates the ATPase activity of ABCB1 by phosphorylation a single serine S671 (Ahmad et al., 1994).

Additional support for the close interaction between ABCB1 and PKC comes from studies where several MDR cell lines overexpress ABCB and PKC, that finally result in a more than 20-fold enhancement of ABCB1 phosphorylation (Blobe et al., 1993). Additionally, decreased PP2A expression and activation was also measured in MDR cells (Ratnasinghe et al., 1998). This suggests that there is a complex process by which PKC/As together with PP2A differentially de/phosphorylate specific serines in the linker region, which may shuttle the protein between different states of ATPase and drug-binding functions.

Moreover, the linker region of ABCBs shares features similar with the R domain of CFTR (cystic fibrosis transmembrane regulator), a structurally related member of ABCBs. This domain has been directly implicated in the regulation of chloride channel activity by PKA (Cheng et al., 1991; Mense et al., 2006; Rich et al., 1991) where dysfunction leads to cystic fibrosis. This supports the possibility that

the linker region maybe functionally equivalent to the R domain and is directly involved in regulating the activity of ABCBs.

Several studies in different organisms reveal the phosphorylation-dependent regulation of ABCBs, however, almost nothing is known about their mechanism in plants. A proteomics approach predicts three possible phosphorylation sites in the linker of *Arabidopsis* ABCB1 (S631 – a putative PKA site), ABCB4, ABCB11 and ABCB21 (Nuhse et al., 2004).

Taking the different investigations into account and considering several indirect conclusions, a regulation of the ABCB-mediated auxin transport by phosphorylation becomes very likely. For example: many lines of evidence show that PIN2 regulates basipetal auxin transport in root epidermal cells in order to trigger gravitropic responses. However *rcn1 pin2* double-mutant analyses indicate that elevated basipetal transport in *rcn1* is NPA sensitive and does not require PIN2. As a consequence the regulation by a NPA-binding protein becomes more likely (Rashotte et al., 2001). Genetic analysis showed that *rcn1abcb1abcb19* seedlings exhibit agravitropic root growth, that is more severe than the phenotype of either *rcn1* or the *abcb1abcb19* double mutant (Figure 8f) (Mravec et al., 2008). This is consistent with the ABCB transporters, ABCB1 and ABCB19, being targets of PP2A dephosphorylation.

Additionally, transport assays have been used to demonstrate decreased polar auxin flow in auxin transport mutants such as the *pin1* (Bennett et al., 1996b; Okada et al., 1991). In young plants (19–26 days) carrying strong *pid* alleles, auxin transport was decreased up to 75% compared to the wild-type, a decrease which is significantly stronger than a reduction of 40-50% in *pin1* mutants. This result indicates that the PID also affects other auxin transport proteins than PIN1.

Finally, four homologous protein kinases from the AGCVIII family, D6 protein kinases (**D6PKs**), affect auxin transport that correlate lateral root initiation, root gravitropism, and shoot differentiation in axillary shoots. Although D6PK co-localizes with PIN proteins on the lower membrane of root cells and D6PK phosphorylates PIN proteins *in vitro* and *in vivo*, this interaction neither effects PIN abundance, tissue distribution, polarity, endocytosis or recycling. Additionally in root epidermal cells D6PK and PIN2 do not co-localize, leading to the suggestion that D6PK has also other phosphorylation targets than PINs (Zourelidou et al., 2009). Based on this it is likely that D6PKs enhance the auxin transport rate by influencing other proteins of the auxin transport complex, such as ABCBs.

3.5.2.9 Kinase/Phosphatase Inhibitors and Activators

As discussed in the last chapter, numerous studies have suggested a correlation between changes of the auxin transport capacity and reversible protein phosphorylation. Additional attempts have been made to correlate the degree of phosphorylation of ABCBs with its efflux activity. And in general, an increase in protein kinase activity and/or phosphorylation of ABCBs has been associated with increased levels of multidrug resistance. Many of these studies involved the use of activators and/or inhibitors of protein kinases/phosphatases to modulate the state of phosphorylation of the different transport proteins. Advantages and drawbacks of the different available inhibitors will be discussed below.

Staurosporine – a protein kinase inhibitor

Staurosporine is an alkaloid isolated from microbial sources. It is known as one of the most potent kinase inhibitors (PKA: IC_{50} 60nM; PKC: IC_{50} 30nM), but unfortunately it is unspecific and multiple cellular effects must be taken into account. Staurosporine directly interacts with ABCB1, most probably at its ATP binding site in the second NBD. This binding is responsible for inhibition of drug interaction, inhibition of ATP hydrolysis and a decreased multi-drug-resistance (Castro et al., 1999; Conseil et al., 2001). Furthermore, staurosporine may also affect ABCB1 gene expression (Chaudhary and Roninson, 1992; Kim et al., 1997). However, based on several observations, staurosporine can reduce the ABCB1-mediated transport by mechanism dependent and independent of PKC phosphorylation.

A recent publication suggests PID as a primary target of staurosporine inhibition in the PIN2-mediated basipetal auxin transport and root gravitropism (Sukumar et al., 2009). Here, application of a low concentration of staurosporine to wild type seedlings phenocopies the reduced basipetal auxin transport and delayed gravitropic response of *pid,* whereas *pid* seedlings are insensitive to the effect of staurosporine on root gravitropism. PIN2 is one of the efflux carriers that mediates basipetal auxin transport and *pin2* loss-of-function induces an drastic agravitropic phenotype that is also insensitive to staurosporine. Finally, *pid* and staurosporine induce a internalization of PIN2, but not PIN1, into endomembrane structures, indicating that staurosporine acts specifically on the PID-PIN2 pathway mediating basipetal auxin transport and root gravitropism (Sukumar et al., 2009). This conflicts with an earlier publication which indeed reports a staurosporine induced inhibition on root gravitropism, but no effect on the basipetal auxin transport nor an effect on the DR5::GUS pattern (Shin et al., 2005). This problem might be due to concentration differences in the various studies (100nM vs 10uM). Additionally, application of staurosporine to suspension-cultured tobacco cells showed an increased accumulation of NAA, but not 2,4-D (Delbarre et al., 1998).

Chelerythrine – a protein kinase inhibitor

Chelerythrine was identified as a potent and selective inhibitor of PKCs (IC_{50} 0.7uM) (Herbert et al., 1990). A publication in 1998 could not confirm the inhibitory effect of chelerythrine on PKCs but reported a lot of additional cellular effects (Lee et al., 1998). However, in general chelerythrine is believed to be a potent inhibitor of PKCs that increases cellular drug accu-mulation (Chambers et al., 1992; Germann et al., 1996). Like staurosporine, chelerythrine also seemed to inhibit transport activity of ABCB1 dependent and independent of phos-phorylation events mediated by PKC by binding to the transporter itself (Castro et al., 1999).

Phorbole Ester – a protein kinase activator

Phorbole ester is a potent tumor promoter in animal cells and is been known for its stimulating effect on protein phosphorylation by activating PKCs (Baudouin et al., 2002; Chambers et al., 1992).

Cantharidin – a protein phosphatase inhibitor

Interestingly, cantharidin was found to have a strong reversal effect on multi-drug resistance and new findings indicate that ABCB1 transcription is inhibited by cantharidin in tumor cells (Zheng et al., 2008).

Cantharidin preferentially inhibits PP2A (MacKintosh and MacKintosh, 1994) and treatment of wild type plants with cantharidin phenocopies *rcn1*, a mutant with reduced PP2A activity, by inducing a delayed gravitropic response that is due to en elevated basipetal auxin transport (Deruere et al., 1999; Muday and DeLong, 2001; Rashotte et al., 2001). Cantharidin treatment and *rcn1* phenotypes are reversible by low NPA concentrations. Application of cantharidin to *pid* mutant seedlings rescues the impaired gravitropism illustrating the reciprocal regulation of RCN/PP2A and PID (Sukumar et al., 2009). Additionally, low doses of cantharidin mimic the *rcn1* phenotype, whereas higher concentrations mimic *rcn1 pp2a* and *rcn1 pp3a* double mutants (Zhou et al., 2004).

Quercetin – an endogenous protein kinase inhibitor

Flavonoids are routinely used as anti-carcinogenic drugs and potent phosphorylation inhibitors in the clinical research field. The widespread use of micromolar concentrations of flavonoids as kinase inhibitors, like PKCs (Agullo et al., 1997; Gamet-Payrastre et al., 1999; Senderowicz, 2000), makes it likely that quercetin also mediates PKC orthologues in plants, like PID and WAG kinases, respectively, in order to regulate auxin transport and plant development. Further, non-flavonoid kinase inhibitors can also displace NPA from microsomal membranes (Bernasconi et al., 1996) suggesting that flavonoids modulate auxin transport at these and other sites by altering protein phosphorylation (DeLong et al., 2002). As discussed in the last chapter, ABCB activity is regulated by phosphorylation and also phosphorylation modulation of ABCBs might be influenced by flavonoids.

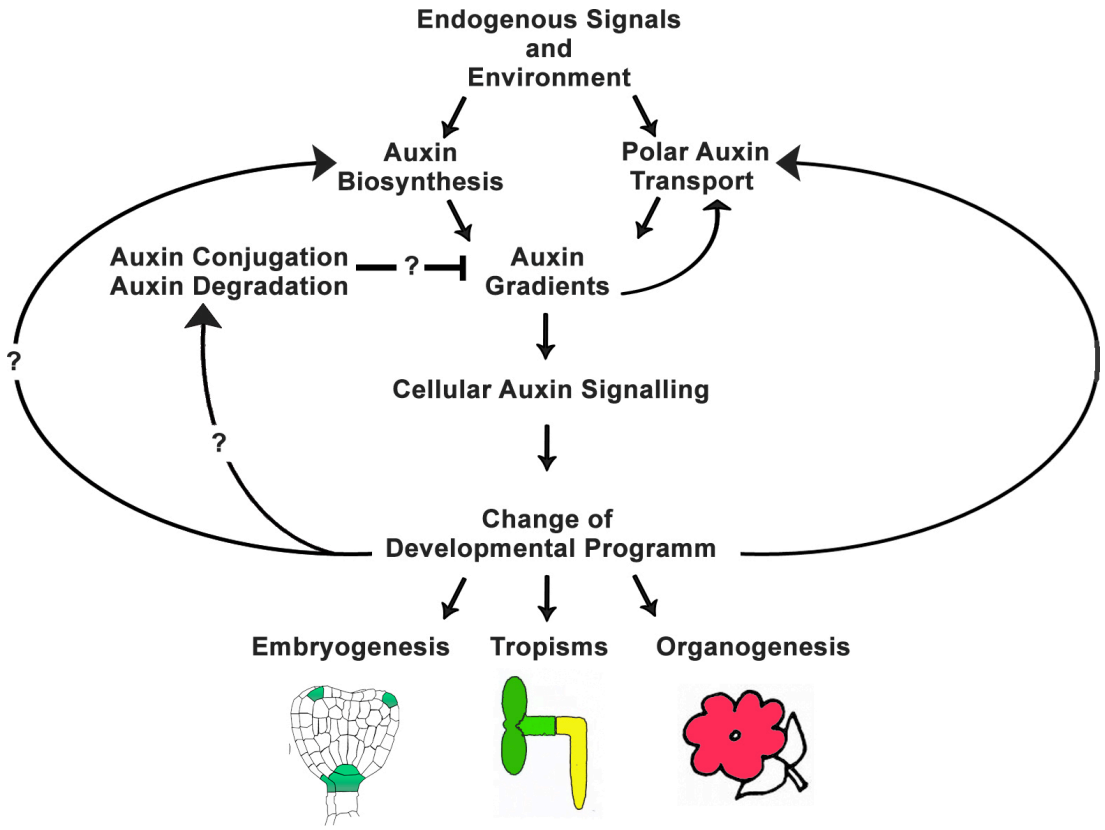


Figure 13. Overview of the Auxin Feedback Regulation

Endogenous and environmental signals modulate auxin biosynthesis and polar auxin transport. Auxin biosynthesis and intercellular transport, presumably in concert with auxin conjugation and degradation processes, determine auxin levels in individual cells and thus auxin distribution patterns within tissues. As a result of auxin accumulation, auxin signaling, triggers predefined changes in developmental programs by activating expression of specific gene sets, including those coding for components of auxin transport and auxin synthesis. In addition auxin signaling provides feedback that regulates the rate and polarity of its own transport. (Modified from Vanneste et. a. 2009).

4 AIM OF THIS WORK

The present work tries to answer some of the unknown regulatory mechanisms during auxin transport:

- Understand the regulatory effect of flavonoids on PIN protein expression and therefore on the regulation of gravitropic bending of the root tip.
- Identify and test the effect of a novel auxin transport inhibitor, BUM, *in vitro* and *in vivo* for its potential to dissect PIN- and ABCB-mediated auxin transport.
- Investigate the role of reversible protein phosphorylation on the auxin efflux machinery. Verify the effect of phosphorylation by the AGC kinase, PINOID, on the transport activity of efflux proteins. Test the role of flavonoids as endogenous regulators of PINOID.

5 Results

5.1 Flavonoids Redirect PIN-mediated Polar Auxin Fluxes during Root Gravitropic Responses

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Abstract

The rate, polarity and symmetry of the flow of the plant hormone auxin are determined by the polar cellular localization of PIN-FORMED (PIN) auxin efflux carriers. Flavonoids, a class of secondary plant metabolites, have been suspected to modulate auxin transport and tropic responses. Nevertheless the identity of specific flavonoid compounds involved and their molecular function and targets *in vivo* are essentially unknown. Here we show that the root elongation zone of agravitropic *pin2/eir1/wav6/agr1* has an altered pattern and amount of flavonol glycosides. Application of noninhibitory concentrations of flavonols to *pin2* roots is sufficient to restore root gravitropism. By employing a quantitative cell-biological approach, we demonstrate that flavonoids restore the formation of lateral auxin gradients in the absence of PIN2. Chemical complementation by flavonoids strictly correlates with an asymmetric distribution of the PIN1 protein. *Pin2* complementation does not result from inhibition of auxin efflux, as supply of the auxin transport inhibitor *N*-1-naphtylphtalamic acid failed *per se* to restore *pin2* gravitropism. We propose that flavonoids promote asymmetric PIN shifts upon gravity stimulation, thus redirecting basipetal auxin streams necessary for root bending.

Own contribution

Figure 3: Exogenous NPA does not rescue the agravitropic response of *pin2*
(A) Gravitropic Assays of wild type and *pin2* roots in the presence of NPA
(B) Kinetics of root bending of *pin2* root upon NPA treatment compared to the wild type

Table 2: The majority of quercetin-treated *pin2* roots form IAA gradients upon gravity stimulation
Quantification of IAA gradients by confocal imaging of DR5-GFP fluorescent signals.

Figure 4. Flavonoid-dependent rescue of *pin2* agravitropic phenotype requires PIN1 and is correlated with asymmetric PIN1 distribution across gravity-stimulated tissues.
(A) Measurements of gravity responses of wild-type, *pin2pin3pin7* and *pin1pin2* roots in the presence of quercetin.

Table S1: The majority of quercetin-treated *pin2* roots form IAA gradients upon gravity stimulation.
Quantification of IAA gradients by confocal imaging of DR5-GFP fluorescent signals.

Table S2: Weak asymmetric IAA gradients were occasionally observed in vertically oriented *pin2* roots.
Quantification of IAA gradients by confocal imaging of DR5-GFP fluorescent signals.

[2] Sharing corresponding authorship.

Supplemental Material can be found at:
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Flavonoids Redirect PIN-mediated Polar Auxin Fluxes during Root Gravitropic Responses*[S]

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The rate, polarity, and symmetry of the flow of the plant hormone auxin are determined by the polar cellular localization of PIN-FORMED (PIN) auxin efflux carriers. Flavonoids, a class of secondary plant metabolites, have been suspected to modulate auxin transport and tropic responses. Nevertheless, the identity of specific flavonoid compounds involved and their molecular function and targets *in vivo* are essentially unknown. Here we show that the root elongation zone of agravitropic *pin2/eir1/wav6/agr1* has an altered pattern and amount of flavonol glycosides. Application of nanomolar concentrations of flavonols to *pin2* roots is sufficient to partially restore root gravitropism. By employing a quantitative cell biological approach, we demonstrate that flavonoids partially restore the formation of lateral auxin gradients in the absence of PIN2. Chemical complementation by flavonoids correlates with an asymmetric distribution of the PIN1 protein. *pin2* complementation probably does not result from inhibition of auxin efflux, as supply of the auxin transport inhibitor *N*-1-naphthylphthalamic acid failed to restore *pin2* gravitropism. We propose that flavonoids promote asymmetric PIN shifts during gravity stimulation, thus redirecting basipetal auxin streams necessary for root bending.

The plant hormone auxin (3-indolyl acetic acid, IAA)[5] controls virtually all plant developmental and physiological processes. In roots, the differential growth response associated with gravity stimulation (gravitropism) occurs in the elongation zone (1, 2) and is a result of the asymmetric distribution of auxin to the lower side of epidermal cells (3). In these tissues accumulating auxin, cell elongation is inhibited and the root tip bends downwards. This cell-to-cell or polar auxin transport (PAT) is determined by the asymmetric cellular localization of auxin in- and efflux components of the ABCB/PGP/MDR, AUX1/LAX, and PIN-FORMED (PIN) family (4–7). Although ABCBs are apparently involved in long-range auxin transport and movements of auxin out of apical regions (8–10), AUX1 and PIN2/*EIR1*/*WAV6*/*AGR1* have been demonstrated to channel auxin from the lateral root cap basipetally to the expanding epidermal cells (11–13).

The regulation of auxin transport during root gravitropic responses is still largely unclear. Among various possible mechanisms, the localized synthesis and directed transport of flavonoids, plant-specific phenylpropanoid compounds, have been shown to modulate the rate of the gravity response (14, 15). A number of lines of experimentation have suggested that flavonoids may act as non-essential auxin transport inhibitors (16–20). This is mainly based on the finding that flavonoids displace binding of synthetic auxin transport inhibitors, like *N*-1-naphthylphthalamic acid (NPA), a herbicide (Naptalam®), *in vitro* (31, 50–52). Moreover, roots of *transparent testa* (*tt*) *Arabidopsis* mutant with manipulated flavonoid levels exhibit altered gravitropic curvature and auxin transport, which are restored to the wild-type level by exogenous application of flavonoids (16, 21). Nonetheless, the identity of the specific flavonoid compounds involved, their molecular targets as well as their mode of action *in vivo* are essentially unknown. Several lines of evidence suggest that ABCBs are directly (8–10, 22, 23) or indirectly (24) regulated by aglycone flavonols. High NPA concentrations cause inhibition of auxin efflux catalyzed by ABCB1/PGP1, ABCB19/PGP19/MDR1 (22, 23), and ABCB4/PGP4/MDR4 (9) (hereafter referred to as ABCBs), most probably by binding to the transporter itself (25). This is in analogy to flavonoids, functioning as inhibitors of plant (22, 23, 26) and mammalian ABCBs (27), probably by mimicking ATP and competing for ABCB nucleotide-binding domains (28). In contrast, the expression and subcellular location of PIN auxin efflux carriers is thought to be a consequence of flavonoid-mediated alteration of auxin concentrations (18, 19). In a pioneer study evidence that flavonoids are functioning as endocrine effectors that specifically

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S3 and Tables S1 and S2.

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[5] The abbreviations used are: IAA, 3-indolyl acetic acid; PAT, polar auxin transport; NPA, *N*-1-naphthylphthalamic acid; EZ, elongation zone; RT, root tip; HPLC, high performance liquid chromatography; DPBA, diphenylboric acid 2-aminoethyl ester; MS, mass spectrometry; GFP, green fluorescent protein.

determine individual PIN gene expression and protein localization was provided (18).

Here, we report that agravitropic loss-of-function mutant *pin2/eir1/wav6/agr1* has impaired patterns of flavonol glycosides. We found that nanomolar concentrations of exogenous flavonols, which have apparently only a mild inhibitory effect on root elongation and gravitropic response in wild-type plants, can partially rescue the agravitropic phenotype of *pin2* roots by promoting asymmetric PIN1 shifts, re-establishing polar auxin fluxes.

EXPERIMENTAL PROCEDURES

Chemicals—The following substances were obtained as indicated MeCN (HPLC Supra grade, Scharlau, E-Barcelona), HCOOH (Fluka, Puriss, Switzerland), methanol (MEOH, Fisher Scientific, UK), HPLC-grade acetonitrile (Fisher Scientific, UK), and H_3PO_4 (Applichem, Germany). Water was purified with a MilliQ Gradient apparatus (<5 ppb, Millipore, Milford, MA). Diphenylboric acid 2-aminoethyl ester (DPBA, Sigma, Germany), NPA (Fluka, Germany), kaempferol (Calbiochem, La Jolla, CA), and quercetin (Fluka, Germany) were dissolved in 100% dimethyl sulfoxide.

Growth Conditions and Plant Material—Seeds were surface sterilized for 5 h in a chamber containing vaporous HCl and sodium hypochlorite and stratified in a 0.1% agar solution for 2 days at 4 °C. Subsequently, the seeds were plated on sterile half-strength MS medium at pH 5.7 containing 2% sucrose solidified with 0.6% phytagel (Sigma), and vertically grown at 22 °C with a 16-h/8-h light/dark cycle. The mutant alleles used in this study were *pin2-1* (29) and *eir1-4* (30).

Flavonoid Fluorescence Staining—Flavonoid compound locations were visualized *in vivo* by the fluorescence of flavonoid-conjugated DPBA compounds after excitation with blue light. Plants were grown for 5 days before staining. Fluorescent staining of whole seedlings was performed according to Buer and Muday (14). Fluorescence was achieved by excitation with fluorescein isothiocyanate filters (450–490 nm, suppression long pass 515 nm) on a Leica DMR fluorescence microscope and ×10 or 20 objectives. Digital images were captured with a Leica DC300 F charge coupled device camera.

Extraction of Phenolic Compounds and HPLC Analysis—Excised roots were incubated overnight in the dark at 4 °C in 0.5 ml of 80% (v/v) methanol (MeOH), extracted, and centrifuged at 18,000 × *g* for 10 min. The supernatant was concentrated to dryness and resuspended in 0.1 ml of 80% MeOH. Aliquots (50 μl) were analyzed by a reverse-phase HPLC (Gynkotek, Germany). Absorbance spectra were recorded with a UVD340S diode array detector (Dionex, Switzerland). Data integration analysis was conducted using the Chromeoleon software (version 6.4, Dionex, Switzerland). The peak height was quantified at 330 nm. A calibration curve for kaempferol was used as reference for single peak quantification. All analyses were performed with at least three independent replicates, each representing 100 roots. Chromatographic conditions were Nucleosil 100-5 C_{18} column (5 μm, 2 × 250 mm, Macherey-Nagel, Düren, Germany); flow rate 1.00 ml min^{-1}, gradient (step, time, %B over A) 1, 25 min, 10–25%; 2, 10 min, 25–70%). Solvent A was H_2O, 0.1% (v/v) H_3PO_4 and solvent B was MeCN.

Structural Elucidation: HPLC-ESI-MS/MS Analysis—HPLC-MS analyses were performed on an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA) fitted with a HTS PAL autosampler (CTC Analytics, Zwingen, Switzerland), an Agilent 1100 binary pump, and an Agilent 1100 photodiode array detector. Chromatographic conditions were Nucleosil 100–3 C_{18} column (3 μm, 2 × 250 mm, Macherey-Nagel, Hoerdt, France); flow rate 0.170 ml min^{-1}, gradient (step, time, %B over A) 1, 25 min, 10–25%; 2, 10 min, 25–70%). Solvent A was H_2O, 0.1% (v/v) HCOOH and solvent B was MeCN, 0.1% (v/v) HCOOH. The HPLC was connected to a Bruker ESQUIRE-LC quadrupole ion trap instrument (Bruker Daltonik GmbH, Bremen, Germany), equipped with a combined Hewlett-Packard Atmospheric Pressure Ion source (Hewlett-Packard Co., Palo Alto, CA). The HPLC output was directly interfaced to the ESI ion source. The MS conditions were: nebulizer gas (N_2) 40 p.s.i., dry gas (N_2) 9 liters/min, dry temperature 300 °C, HV capillary 4000 V, HV EndPlate offset −500 V, capillary exit −100 V, skimmer1 −28.9 V, and trap drive 53.4. The MS acquisitions were performed in the negative electrospray ionization mode, at normal resolution (0.6 unit at half-peak height), under ion charge control conditions (10,000) in the mass range from *m/z* 100 to 1000.

The MS^2 acquisitions were obtained in the auto-MS/MS mode. The isolation width was 4 units, the fragmentation cut-off set by "fast calc," and the fragmentation amplitude set at 0.9 V in the "SmartFrag" mode. The total amounts of flavonoid compounds were calculated as the sum of the areas ($\times 10^6$ arbitrary unit) of the mass signals identified during HPLC-ESI-MS analysis. Each extraction consists of a pull of 100 different roots. Quantification of RT-EZ flavonoid compounds is the result of two independent extractions in which each time 150 5-mm long root apices from 12 different agar plates were pulled together.

Gravitropic Assays—4-Day light-grown seedlings were transferred from control plates to plates containing nutrient media optionally supplemented with quercetin or kaempferol (100 or 200 nM) or NPA (100 nM to 5 μM). After 24 h of adaptation and growth in the new media, plates were turned 90°.

In one type of gravitropic assay, after 24 h of growth under gravistimulation, seedlings were scanned using Epson Perfection photo 2450 and angles of gravitropic curvature were measured from digital pictures using the tool "Image Manager" of the Leica IM1000 software (Leica, Heerbrug, CH). Each gravity stimulated root was assigned to one of 12, 30° sectors; the length of each bar represents the percentage of seedlings showing the same direction of root tip growth. To enable direct comparison of root bending, percentual occurrence of 60 and 90° bending (sum of 60 and 90° sectors), the dominant bending sectors of wild-type roots under control conditions (98.4%), was defined as *relative root bending*.

Short pulses of gravity stimulation were achieved by turning the plates 90° for 2 h, which corresponds to the peak of gravity-induced flavonoid accumulation (14). After 2 h of gravity stimulation, roots were excised and flavonoids extracted as described, or expression of $DR5_{rev}$-GFP reporter protein analyzed on a Leica TCS SP2 CLSM.

Kinetics of root bending were performed and analyzed as described in Ref. 31. All gravitropic assays were performed in the dark to prevent phototropic responses. In some cases (Table 2 and supplemental Figs. S1 and S2), angles of gravitropic curvature were measured in a blind assay, to reduce possible unbiased calculations.

Immunocytochemistry—PIN1 immunolocalization was performed as previously described (32) with PIN1 specific antibody (33) at 1:1000 dilution and anti-rabbit Cy3-conjugated secondary antibodies. Confocal imaging of Cy3 and *DR5rev::GFP* was carried out on a Leica SP2 AOBS microscope. In some cases (Fig. 4*C* and supplemental Fig. S3), symmetries of DR5-GFP gradients and PIN1 distribution were measured in a blind assay, to reduce possible unbiased calculations.

Data Analyses—Statistical analysis was performed using SPSS 11.0 (SPSS Inc., Chicago, IL).

RESULTS

pin2 Roots Have an Altered Flavonoid Pattern—*pin2/eir1/wav6/agr1 Arabidopsis* mutant (referred to as *pin2* hereafter), one of the best characterized auxin transport mutants, exhibits reduced basipetal auxin transport and agravitropic root growth (2, 29, 30, 34). As a starting point of this work, we investigated whether defects in basipetal auxin transport in *pin2*, which result in agravitropic responses (35), are linked to an altered accumulation of specific endogenous flavonoids and whether flavonoids could be directly implicated in the control of the gravitropic responses. DPBA, a fluorescent dye that specifically interacts with flavonoids, allows *in situ* flavonoid staining and localization in *Arabidopsis* seedlings (18, 20, 36). In wild-type seedlings, flavonoid DPBA staining is restricted to the shoot apex and cotyledons, the root-shoot junction, along the primary root, and most intensely to the root elongation zone (Fig. 1*A* and supplemental Fig. S1, *A–C*) (36). In contrast, flavonoid-DPBA fluorescence in the *pin2* mutant was clearly lower at the root tip-elongation zone (RT-EZ) (Fig. 1*B* and supplemental Fig. S1, *E–G*). Manipulation of endogenous auxin levels by addition of 100 nM IAA increased DPBA fluorescence in the wild type (14) and, although to a lesser extend, also in the *pin2* RT-EZ (supplemental Fig. S1, *D* and *H*), suggesting that auxin and flavonoid levels *in planta* are interconnected (18, 36).

To determine how flavonoid distribution was affected by PAT alterations, we qualitatively and quantitatively investigated endogenous flavonoid derivatives present in wild-type and *pin2* RT-EZ and entire roots using HPLC-UV-(−)-ESI-MS and HPLC-ESI-MS/MS, respectively (Table 1). Consistent with DPBA staining profiles (Figs. 1, *A* and *B* and supplemental S1), we found that the total amount of flavonoids was significantly reduced in the RT-EZ of *pin2* mutant (Fig. 1, *E* and *G*), whereas no significant difference was observed over the entire root (Figs. 1*E* and supplemental S2). *pin2* roots showed altered accumulation of specific flavonol glycosides both in the RT-EZ and in the entire root (Table 1 and *arrows* in the extraction ion chromatograms of the masses of interest in Figs. 1*G* and supplemental S2). In *pin2* entire roots and RT-EZ, a shift from di- and triglycosylated flavonols to monoglycosylated flavonols, such as K-G-3 (compound **18**), was observed (Table 1), which suggests that auxin levels may have an effect on the expression or activity

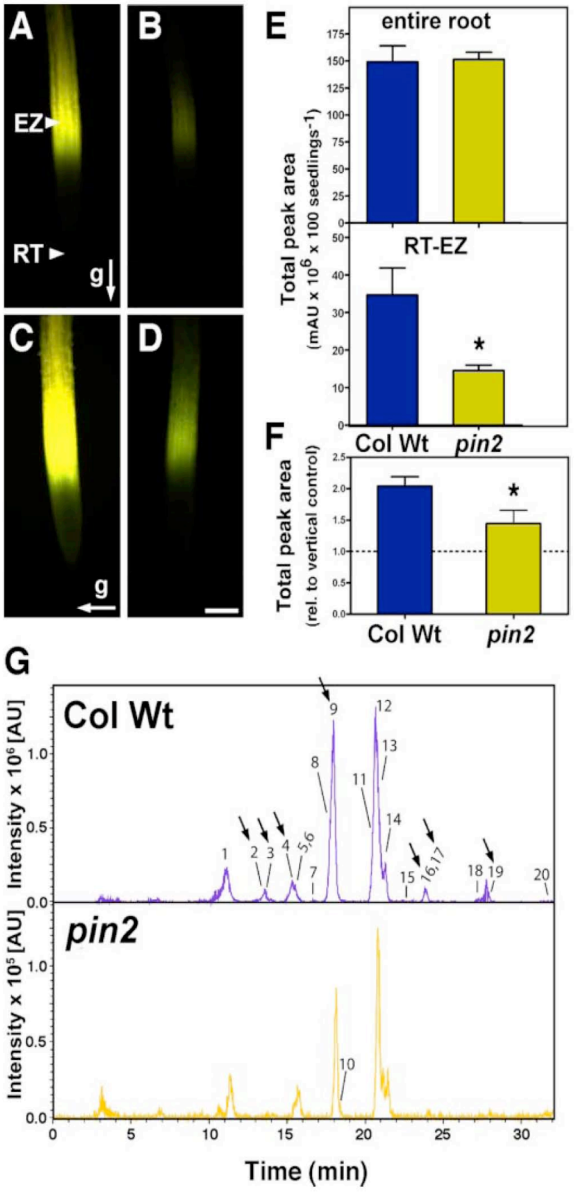


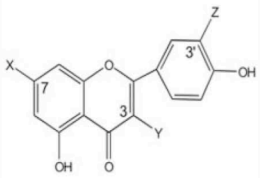
FIGURE 1. **Defects in basipetal auxin transport are associated with altered root flavonoid accumulation.** *A–D*, flavonoid accumulation in the entire root and root elongation zone of wild type (*Col Wt*; *A* and *C*) and *pin2* (*B* and *D*). Accumulation of flavonoids in control and 2-h gravity-stimulated roots visualized *in situ* using DPBA (*yellow* fluorescence) as described under "Experimental Procedures." The *arrows* indicate the direction of the gravity vector relative to the root. *Bar*, 100 μm. *E*, total amount of flavonoid derivatives detected in the entire root and RT-EZ of wild type and *pin2*. *F*, gravity-induced root phenolic compound accumulation normalized to phenolic compound accumulation in vertical control. Values represent mean ± S.E. ($n = 2–5$ replicates); *, significantly different from the wild type (Student's *t* test, $p < 0.05$). *G*, representative sum of extracted ion chromatograms $[M - H]^-$ of flavonoid derivatives found in wild type and *pin2* RT-EZ analyzed by HPLC-ESI-MS. Significantly altered compounds are indicated by *arrows*. Peak numbers correspond to flavonoid derivates listed in Table 1. Note the 10 times lower intensity scale for *pin2* root elongation zone in comparison to wild type.

of corresponding glycosyltransferases. This is supported by *in silico* (www.genevestigator.ethz.ch) expression analysis of two glycosyltransferase genes that are involved in flavonoid biosynthesis in *Arabidopsis* (37). At5g17050, which encodes for a fla-

TABLE 1

Flavonoid derivatives detected in the MeOH extracts of entire root and RT-EZ of wild type and *pin2*

The identification of the flavonoid derivatives was achieved by HPLC-UV(−)ESI-MS/MS, comparison to reference compounds was according to Refs. 46–49. Each compound was quantified by integration of the corresponding signal area ($\times 10^6$ arbitrary units (AU)) presented in the extract ion chromatogram (EIC, $[M-H]^-$) after HPLC-MS experiment. Values of entire roots represent mean ± S.E. of at least three independent experiments (Student's *t* test, $p < 0.05$, $n \leq 2-4$). Each extraction consists of a pool of 100 different roots. Quantification of RT-EZ flavonoid compounds is the result of at least two independent extractions in which each time 150 5-mm long root apices from 12 different agar plates were pooled. The natural flavonoid derivates described have abbreviations as follows: G, glucose; K, kaempferol; I, isorhamnetin; Q, quercetin; R, rhamnose. Numbers indicate the position of glycosylation relative to the flavonol core. All flavonols identified are *O*-glycosylated.



	compound	Rt (min)	MW	trivial name	entire root (Area x 10^6 AU)		RT-EZ (Area x 10^6 AU)	
					Col	*pin2*	Col	*pin2*
	2	13.6	756	Q-R-G-3-R-7	5.9 ± 0.47	3.7 ± 0.4	1.25 ± 0.2	0.2 ± 0.1
	3	14.2	610	K-G-3-G-7	1.7 ± 0.32	1.2 ± 0.3	0.2 ± 0.1	0.1 ± 0.0
	4	15.5	740	K-R-G-3-R-7	10.1 ± 0.51	9.0 ± 0.7	1.3 ± 0.2	0.4 ± 0.1
	5	15.8	772	Q-G-G-3-R-7	1.2 ± 0.05	1.5 ± 0.1	0.2 ± 0.1	0.1 ± 0.0
	7	16.8	594	K-R-3-G-7	0.7 ± 0.15	0.2 ± 0.03	0.1 ± 0.03	< 0.1
	8	17.8	756	Q-G-R-R	2.3 ± 0.25	0.9 ± 0.2	0.3 ± 0.1	< 0.1
	9	18.1	610	Q-G-3-R-7	52 ± 4.1	43.5 ± 1.0	15.6 ± 3.5	5.3 ± 0.7
	10	18.3	756	K-G-G-3-R-7	n.d.	1.1 ± 0.2	n.d.	< 0.1
	12	20.8	594	K-G-3-R-7	49.8 ± 7.7[1)]	41.5 ± 3.7	12.03 ± 2.5	6.15 ± 0.2
	13	21.1	594	Q-R-3-R-7				
	14	21.3	624	I-G-3-R-7	16.7 ± 4.3	20 ± 2.3	2.5 ± 0.4	1.5 ± 0.1
	15	22.5	610	Q-R-G-3	0.7 ± 0.3	0.6 ± 0.13	0.1 ± 0.0	n.d.
	16	23.9	464	Q-G-3	1.5 ± 0.1	2.0 ± 0.4	0.3 ± 0.1	0.1 ± 0.0
	17	24	578	K-R-3-R-7	3.8 ± 0.3	1.1 ± 0.1	0.6 ± 0.1	0.3 ± 0.1
	18	27.4	448	K-G-3	1.7 ± 0.1	12.1 ± 1.6	0.2 ± 0.1	0.4 ± 0.1
	19	28	478	I-G-3	1.3 ± 0.1	2.6 ± 0.6	0.1 ± 0.0	< 0.1
	20	32.8	432	K-R-3	1.0 ± 0.2	1.3 ± 0.2	0.1 ± 0.0	< 0.1
non flavonoids	1	11.5	448	Glucobrassicin	5.6 ± 0.8	6.9 ± 0.8	4.7 ± 1.2	6.0 ± 1
	6	15.8	478	4-Methoxyglucobrassicin	5.3 ± 1.3	5.4 ± 1.1	2 ± 0.1	2.2 ± 0.9
	11	20.3	478	Neoglucobrassicin	42.3 ± 6.4	26 ± 5.0	8.8 ± 1.2	4.0 ± 2

[1] Compounds 12 are 13 are co-eluting in the HPLC-(−)-ESI-MS chromatogram and show the same quasi-molecular ions (*m/z* 593); therefore, they were integrated together.

vonoid 3-*O*-glucosyltransferase, is induced by 1-naphtylacetic acid and 2,4-dichlorophenoxyacetic acid treatment, whereas At4g14090, anthocyanin 5-*O*-glucosyltransferase, is down-regulated by auxin transport inhibitor treatments. Conversely, those peaks, whose accumulation is affected in *pin2* roots, may be functionally important for the regulation of auxin transport during root gravitropism.

As previously reported (14, 38), a 2-h gravity stimulation increased the DBPA fluorescence in wild-type RT-EZ by nearly 2-fold, with a maximum at 1.5 to 2.5 h after stimulation. A smaller but significant increase in DPBA fluorescence was observed also in *pin2* mutant (Fig. 1, *C–D*). Flavonoid quantification by HPLC-UV (Fig. 1*F*) was consistent with the DPBA staining. Collectively, our results demonstrate that the synthesis and transient accumulation of specific flavonoid glycosides in the root tip-elongation zone, but not over the entire root or in the shoot, are impaired quantitatively and qualitatively in *pin2* (Figs. 1, supplemental S1 and S2).

Flavonoids Partially Rescue the Agravitropic Response of pin2 Roots—To test whether flavonoid concentrations play a critical role in the response to gravity stimuli, we searched for conditions in which flavonoids could be supplied without negatively affecting root growth and gravitropism most probably by acting as auxin transport inhibitors (16, 18). Concentrations up to 100 nM kaempferol or quercetin did not significantly influence wild-type gravitropic responses after 24 h (95.5 and 99.0% instead of 98.4% (relative root bending (= sum of 90 and 60° sectors, respectively); see "Experimental Procedures" (Fig. 2*A*)). Moreover, root-bending kinetics demonstrated that treatment with 100 nM quercetin did not significantly alter the bending performance of wild-type roots over 24 h compared with the solvent control (Fig. 2*B*). Importantly, bending of wild-type roots in the presence and absence of quercetin is virtually identical after 2 and 24 h, time points used in this study), however, small but not significant differences are found between 2 and 24 h.

Roots of the *eir1-4* mutant, a severe agravitropic allele of *pin2* (30), were gravity stimulated for 24 h in the presence of 100 nM flavonoids. Intriguingly, *pin2* gravitropic root bending (33.5%) was significantly restored by quercetin (50.0%) and kaempferol (52.1%, Fig. 2, *A* and *B*). Restoration of relative root bending by flavonols was roughly 25% and therefore only partial.

The same gravitropic assay was performed in the presence of the synthetic inhibitor of polar auxin efflux NPA, which blocks basipetal IAA movement from the root tip (39). In wild-type plants, treatment with 5 μM NPA, a concentration routinely used, resulted in an agravitropic phenotype (39.4%) (16, 35), similar to that found for *pin2*. However, NPA failed to restore

Redirection of Auxin Flows by Flavonoids

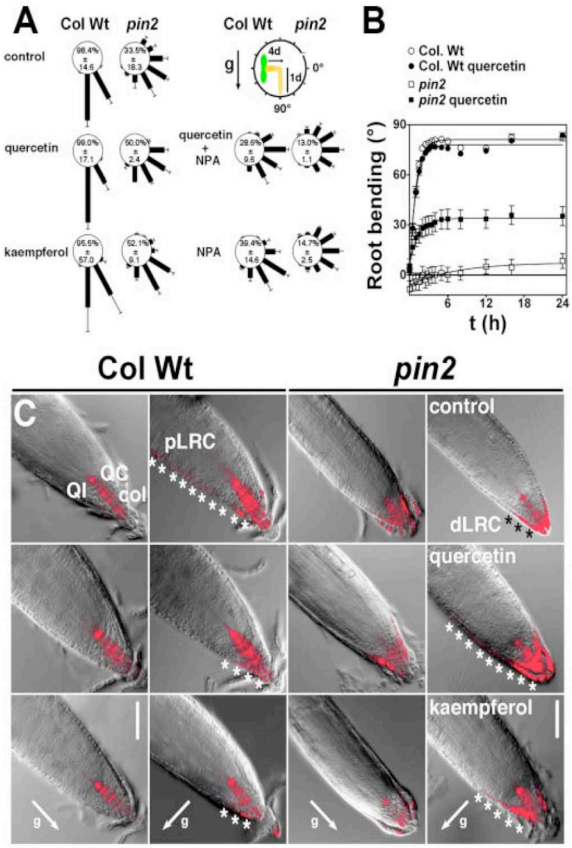


FIGURE 2. **Exogenous flavonols partially rescue the agravitropic response of *pin2* by restoring asymmetric auxin gradients.** *A*, gravity responses of wild-type (*Col Wt*) and *pin2* (*eir1-4*) roots 24 h after reorientation of 90° to horizontal in the presence of the indicated auxin transport inhibitors. Each gravity-stimulated root was assigned to one of 12, 30° sectors (see schematic). The *length of each bar* represents the mean percentages ± S.D. of seedlings showing the same direction of root growth of at least three independent experiments; *numbers* correspond to the mean (±S.D.) percent occurrence of 90 and 60° bending (sum of 90° and 60° sectors). *B*, time series of wild-type (*Col Wt*) and *pin2* (*eir1-4*) root curvature after reorientation of 90° to horizontal in the presence and absence of 100 nM quercetin analyzed as described in Ref. 24). Data are mean ± S.E. ($n = 4$ with 50 seedlings per allele and treatment). *C*, expression of the auxin-reporter construct $DR5_{rev}$-GFP in wild type (*Col Wt*) and *pin2* (*eir1-4*) root tips was assessed prior to, and after 2 h gravity stimulation on control (*top row*), quercetin (*middle row*), and kaempferol-treated roots (*bottom row*). *White asterisks* indicate more pronounced *DR5-GFP* expression at the lower side of gravistimulated roots suggesting enhanced basipetal auxin reflux. The gravity vector relative to the root tip is indicated by an *arrow*. *QC*, quiescent center; *QI*, columella initials; *col*, mature columella cells; *dLRC*, distal lateral root cap; *pLRC*, proximal lateral root cap. *Bar*, 75 μm.

but rather impaired *pin2* root gravitropism (14.7%). To exclude that restoration of gravitropism in *pin2* was only found at lower concentrations or at a particular NPA dose as reported for a phosphatase 2A mutant, *rcn1* (40), we quantified root gravitropism of wild-type and *pin2* after treatment with NPA from 5 μM down to the concentration used with the flavonols (100 nM). Unlike quercetin and kaempferol, over this concentration range NPA had a negative effect on gravitropism and was not able to restore root gravitropism in wild-type nor in *pin2* (Fig. 3). Finally, we performed quercetin treatment in the presence of NPA, and found that in wild-type NPA, quercetin had an additive effect (28.6%), whereas in *pin2* the quercetin action was

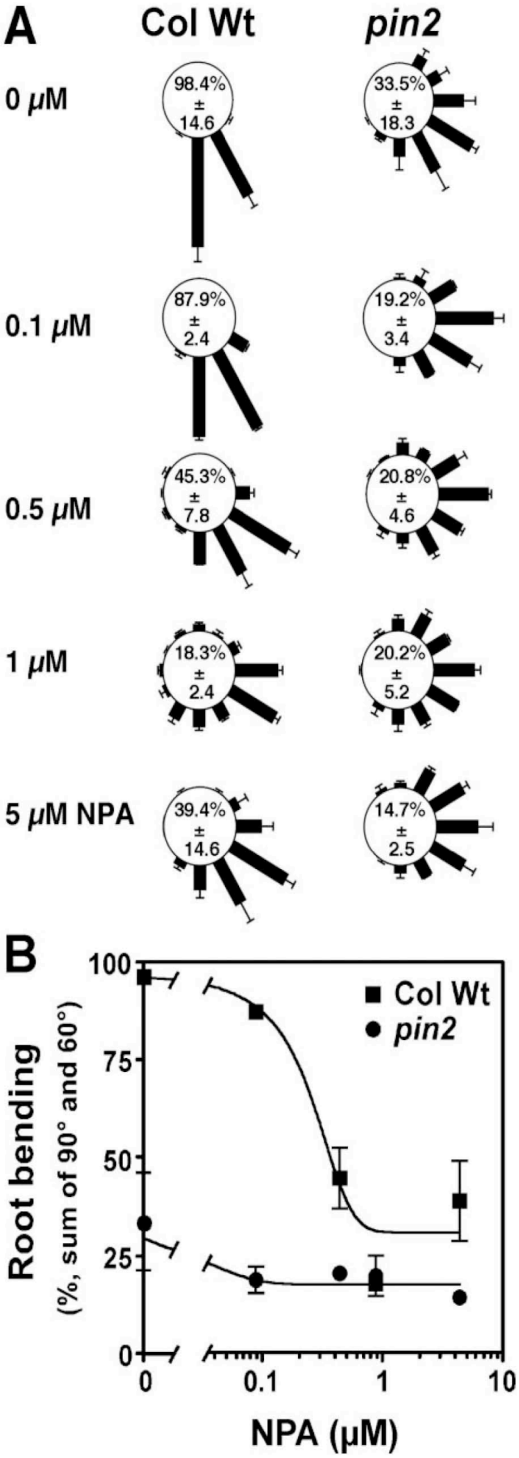


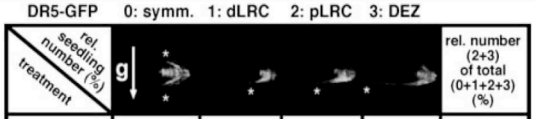
FIGURE 3. **Exogenous NPA does not rescue the agravitropic response of *pin2*.** *A*, gravity responses of wild-type (*Col Wt*) and *pin2* (*eir1-4*) roots 24 h after reorientation of 90° to horizontal in the presence of the indicated concentrations of NPA. Each gravity-stimulated root was assigned to one of 12, 30° sectors (see Fig. 2). The *length of each bar* represents the mean percentages ± S.D. of seedlings showing the same direction of root growth of at least three independent experiments; *numbers* correspond to the mean (±S.D.) percent occurrence of 90 and 60° bending (sum of 90 and 60° sectors). *B*, mean percentages of plants showing 90 and 60° bending (sum of 90 and 60° sectors) ± S.D. of three independent experiments.

TABLE 2

The majority of quercetin-treated *pin2* roots form IAA gradients upon gravity stimulation

Relative numbers of wild type (Wt) and *pin2* seedling showing IAA gradients. Definition of IAA gradients is based on the strength of IAA movement from the LRC to the EZ, as assessed by confocal imaging of DR5-GFP fluorescent signals upon 2-h gravity stimulation. Gradient symmetry is quantified in arbitrary units (0, symmetric signal; 1, weak signal asymmetry, up to distal lateral root cap (dLRC); 2, intermediate signal asymmetry, up to proximal lateral root cap (pLRC); 3, strong signal asymmetry, up to elongation zone (DEZ)). Number of analysed wild-type (*pin2*) seedlings was 60 (65) on solvent control and 105 (100) on 100 nM quercetin plates, respectively. Gravity vector (*g*) relative to the root is indicated by an *arrow*; symmetry of DR5-GFP signals by asterisks. For more details and absolute numbers, see supplemental Table S1.

DR5-GFP 0: symm. 1: dLRC 2: pLRC 3: DEZ



rel. seedling number (%) / treatment		0: symm.	1: dLRC	2: pLRC	3: DEZ	rel. number (2+3) of total (0+1+2+3) (%)
Wt	- Quer	11.7	41.7	43.3	3.3	46.7
	+ Quer	14.3	34.3	41.0	10.5	51.4
pin2	- Quer	53.8	18.5	15.4	12.3	27.7
	+ Quer	21.0	17.0	32.0	30.0	62.0

prevented by NPA (13.0%), indicating that the rescue of *pin2* agravitropic response by quercetin is NPA-sensitive (Fig. 2*A*).

The cause of *pin2* agravitropic root growth is a failure in the accumulation of auxin at the lower side of the root elongation zone (30). We therefore tested whether flavonoid treatment restored the asymmetric auxin distribution necessary for differential growth of epidermal cells during gravitropic responses. This was achieved by monitoring the *DR5:GFP* expression, which reflects relative auxin levels. As previously reported (41), in wild-type seedlings with vertically grown roots, the GFP fluorescence appeared in specific stele cell files and was localized in the quiescent center, in the columella initials, and in the mature columella cells (col) (Fig. 2*C*). Vertically oriented *pin2* roots exhibited a strong signal in the distal lateral root cap with only weak extension toward the proximal lateral root cap, reflecting a defect in basipetal auxin distribution. In vertically oriented roots treatments with 100 nM quercetin or kaempferol resulted in fluorescence essentially similar to the control condition in both the wild-type and *pin2* mutant (Fig. 2*C*). Upon a 2-h gravity stimulation, fluorescent signals in wild-type roots appeared in the lower sides, in the distal lateral root cap and EZ, both in solvent controls (control) and, although slightly reduced, in the presence of flavonoids (Fig. 2*C*, *asterisks*). In contrast, flavonoid treatment of gravity-stimulated *pin2* roots resulted in a gain of a strong asymmetric signal in the entire lateral root cap with significantly increased fluorescence on the lower half of the root and decreased fluorescence on the upper half of the root (Fig. 2*C*, *asterisks*).

To statistically quantify asymmetric auxin accumulation after gravity stimulation, we determined root auxin gradient symmetry (in arbitrary units from 0 to 3) in relation to their tip orientations relative to the future gravity stimulation vector prior to gradient analysis (for details see legends of Table 2 and supplemental Figs. S1 and S2). Compared with wild type (11.7%, Table 2), the majority of *pin2* seedlings tested exhibited a symmetrical auxin distribution (53.8% of seedlings showed auxin gradient symmetry "0"). Only occasionally strong asymmetric auxin gradients (defined as percentage of sum of gradient symmetry "2 + 3") could be observed in *pin2* upon gravity stimulation (27.7%, Table 2), which is in line with the results of the gravitropic assays (Fig. 2*A*). Interestingly, a similar frequency of clear auxin gradients in *pin2* was also observed in vertically grown roots (31.7%) but was not found in the wild type (1.7%, supplemental Table S2). The gain of signal asymmetry between the lower and upper side in flavonoid-treated, gravity-stimulated *pin2* roots continued in the elongation zone and was observed in the majority of the flavonoid-treated roots tested (62.0% compared with 27.7% without treatment, Table 2). In contrast, quercetin treatment had only a negligible effect on wild type (51.4 compared with 46.7% without treatment). Interestingly, also weak auxin gradients apparently result in gravitropic responses in wild-type roots, because inclusion of class 1 roots (Table 2; weak signal asymmetry, auxin gradient up to distal lateral root cap) enhances the relative number of roots showing an asymmetric gradient to 88.3%. This number corresponds to that of the roots exhibiting gravitropic responses (Fig. 2). These data show that exogenous flavonoids are able to re-establish the asymmetric *DR5-GFP* activity in the *pin2* mutant roots building the prerequisite for restoration of gravitropic responses.

Flavonoids Promote Asymmetric PIN1 Shifts—Because some levels of functional redundancy between PIN proteins have been demonstrated (42, 43), we tested the root gravitropic responses of the triple *pin2 pin3 pin7* mutant in the presence of 100 nM quercetin after 24 h. A strong agravitropic root phenotype was evident for *pin2 pin3 pin7* in control conditions (Fig. 4*A*) (43), which could be partially rescued by the application of 100 nM quercetin (47.8 compared with 26.1% without treatment, Fig. 4*A*) or kaempferol (data not shown). From this result, we conclude that neither PIN3 nor PIN7 are required for flavonoid-dependent rescue of the agravitropic response of *pin2*, being in-line with their proposed role and expression in gravity perception tissues (43). Importantly, when we performed the same gravitropic assay with roots of the double mutant *pin1 pin2*, quercetin could not complement its agravitropic phenotype (15.7 compared with 12.2% without treatment, Fig. 4*A*). These data demonstrate that PIN1 is essential for flavonoid-dependent, partial complementation of *pin2* gravitropism, which is in agreement with its expression in gravity transduction or response tissues.

To trace the behavior of PIN1 protein during *pin2* gravitropic responses and to uncover a possible link between the action of flavonoids and PIN1 activity *in vivo*, we analyzed PIN1 localization in *pin2* roots exposed to 100 nM quercetin prior to, and during a 2-h gravity stimulation. Consistent with previous reports (42, 43), in vertically oriented wild-type roots PIN1 was mainly found at the basal (lower) end of vascular and endodermis cells with occasional weak expression in the epidermis and cortex (Fig. 4*B*, *i*). In vertical *pin2* roots, PIN1 was ectopically expressed in the endogenous PIN2 domain, showing symmetric apical (up) localization in the epidermis and basal (down) localization in cortex cells (*arrows* in Fig. 4*B*, *ii*) (42). This symmetric PIN1 location was only rarely altered by a gravity stimulus on solvent control (12.5% of roots showing this pattern, Fig. 4*C*, *i*).

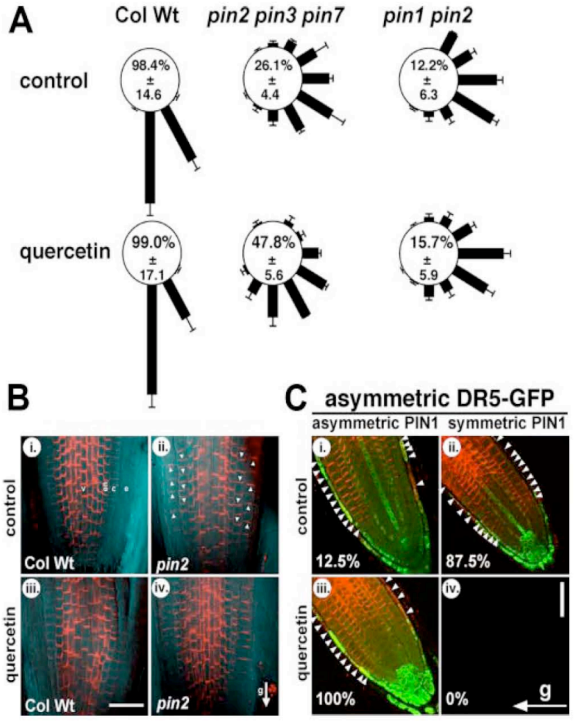


FIGURE 4. **Flavonoid-dependent rescue of *pin2* agravitropic phenotype requires PIN1 and is correlated with asymmetric PIN1 distribution across gravity-stimulated tissues.** *A*, gravity responses of *pin2 pin3 pin7* and *pin1 pin2* (*pin1 eir1*) roots after 24 h in the presence of quercetin. The *length of each bar* represents the mean percentages ± S.D. of seedlings showing the same direction of root growth of at least three independent experiments; *numbers* correspond to the mean (±S.D.) percent occurrence of 60 and 90° bending (sum of 60 and 90° sectors). *B*, whole-mount *in situ* immunolocalization of PIN1 protein (*red*) in 5-day *pin2* (*eir1–4*; *ii* and *iv*) and wild-type (*Col Wt*; *i* and *iii*) vertical seedlings transferred on media supplemented with 100 nM quercetin. Gravity vector is indicated by an *arrow*. *White arrows* indicate PIN1 protein apical localization in the epidermis and basal localization in the cortex cells of *pin2* root tip. Note that the appearance of slightly different PIN1 signals in the *pin2* epidermis and cortex (*ii–iv*) do not reflect unequal expression but are the result of unequal background intensities due to scattered light. *v*, vascular bundle; *en*, endodermis; *c*, cortex; *e*, epidermis. *Bar*, 30 μm. *C*, whole mount *in situ* immunolocalization of PIN1 protein in *pin2* after 2 h of gravity stimulation; gravity vector is indicated by an *arrow*. 4-Day *pin2* seedlings were transferred on media supplemented with 100 nM quercetin or the solvent (control). *Red*, PIN1; *green*, $DR5_{rev}$-GFP expression. *White arrows* indicate more pronounced PIN1 proteins levels at the lower or upper side of the gravity-stimulated root tip. *Bar*, 30 μm. Percentages indicate relative occurrence of asymmetric or symmetric PIN1 distributions with asymmetric DR5-GFP signals; the total number of analyzed roots that showed simultaneous clear DR5-GFP and PIN1 signals was 47.

Treatments with 100 nM quercetin expanded the distribution of PIN1 and slightly its expression as previously reported (18) but did not affect its symmetry of expression neither in wild type nor in *pin2* vertically grown roots (Figs. 4*B*, *iii–iv*). In contrast, gravity stimulation of *pin2* roots in the presence of 100 nM quercetin resulted in asymmetric expression of PIN1 protein, with stronger PIN1-specific signals at the lower side of the root tip (Fig. 4*C*, *iii*). The establishment of flavonoid-mediated PIN1 gradients strictly correlated with the development of asymmetric DR5-GFP signals (100%, Fig. 4*C*, *iii*), whereas in the absence of quercetin asymmetric PIN1 patterns correlating with asymmetric DR5-GFP signals were only rarely found (12.5%, Fig. 4*C*, *i*). We never found asymmetric DR5-GFP gradients that correlated with symmetric PIN1 patterns (0%, Fig. 4*C*, *iv*) or symmetric DR5-GFP gradients correlating with asymmetric PIN1 expression (0%, supplemental Fig. S3, *i*). But for a few roots we found a weak correlation between asymmetric PIN1 patterns and symmetric DR5-GFP (22.2%, supplemental Fig. S3, *iii*).

DISCUSSION

The current paradigm in auxin research is that flavonoids act as non-essential, but important multifunctional, endogenous modulators of PAT by transiently accumulating in the epidermal cells of the root elongation zone (14, 16–19, 36). Our data demonstrate that defects in basipetal auxin transport are associated with altered root flavonoid accumulation. In conditions in which PIN2-dependent auxin transport is genetically blocked, synthesis and transient accumulation of specific flavonoid glycosides in the root tip elongation zone, but not over the entire root or in the shoot, are impaired quantitatively and qualitatively (Figs. 1, and supplementalS1 and S2). However, *pin2* roots retain the ability to accumulate flavonoids in response to a gravity stimulus, but not as efficiently as the wild type.

Application of low concentrations of exogenous flavonoids partially restores gravitropic root tip bending in a genetic background defective in basipetal auxin transport and this mode of flavonoid action is apparently distinct from that of NPA. Moreover, our results showing that gravity stimulation of *pin2* roots in the presence of a wide concentration range of NPA shown to inhibit PAT (24, 35, 40) did not complement *pin2* gravitropism, suggest that in our experimental conditions and concentrations of flavonoids (Fig. 2*B*) predominantly do not act as PAT inhibitors. Quantitative cell biology analysis of DR5-GFP signals show that partial, chemical complementation of gravitropism by exogenous flavonoids is accompanied by re-establishment of asymmetric distribution of *DR5-GFP* in the *pin2* mutant roots. This suggests that restoration of gravitropic responses is achieved via bypassing the requirement of an active PIN2 protein, thus implying the activation of a PIN2-independent mechanism for basipetal auxin transport.

Quantification of root gravitropic response of different *pin* mutant combinations suggests that PIN1 is essential for flavonoid-dependent complementation of *pin2* gravitropism, which is in agreement with its expression in gravity transduction or response tissues. Using a quantitative cell biological approach, we also provide evidence that flavonoid-dependent rescue of *pin2* agravitropism by PIN1 is correlated with asymmetric PIN1 distribution across gravity-stimulated tissues. In summary, our findings suggest that PIN1 is the auxin efflux complex component that facilitates basipetal auxin fluxes for gravitropic responses in flavonoid-treated *pin2* roots. The observed basal-apical PIN1 shifts in *pin2* roots (Fig. 4*B*, *ii*) are in line with the finding that PIN1 and PIN2 have redundant roles in the root meristem size control (43) and that PIN1 can functionally replace PIN2 when ectopically expressed and localized at the upper side of epidermal cells (5). Moreover, PIN1 showed a "PIN2-like" apical localization in epidermis and basal localization in cortex cells in roots of *pin2* mutants (42). However, our data indicate that flavonoids are the native key effectors that promote asymmetric PIN1 shifts with stronger PIN1-specific signals at the lower side of the root tip in response to a gravity

stimulus, thus redirecting basipetal auxin streams necessary for root tip bending.

The fact that flavonols can inhibit PAT and displace NPA from their membrane binding sites has led to the idea that flavonoids and NPA act on similar targets using identical mechanisms. Several lines of evidence suggest that plant ABCBs, like ABCB1, ABCB4, and ABCB19, are direct targets of flavonoid regulation (8, 22, 23) via protein phosphorylation, protein-protein interaction (31), or inhibition of ATPase activity or allosteric binding in analogy to mammalian ABCBs (28). This direct, modulatory role of flavonoids can be widely phenocopied by NPA. However, the effect of flavonoids on members of the PIN family has received less attention and seems to be indirect (transcriptional) and result in activation of auxin streams (18, 20) and cannot be mimicked by NPA.

In summary, with this work we provide two lines of evidence demonstrating that flavonoids, at least under our experimental conditions, do not solely inhibit efflux transportes, but are able to function as versatile modulators of polar auxin flows. First, flavonoid concentrations applied to the roots did not significantly alter over 24 h wild-type gravitropism and therefore most likely also not PAT. Second, NPA over a wide concentration range failed to restore *pin2* root gravitropism, whereas partial rescue of *pin2* agravitropic response by quercetin was NPA sensitive. Our data showing that flavonoids can promote PIN1 shifts in response to a gravity stimulus underline an involvement of flavonoids in cellular trafficking of auxin transport complex components as recently suggested (18, 20).

The partial restoration of gravitropism in *pin2* by flavonoid treatment parallels the previously published complete restoration of basipetal auxin transport (16, 17) and root gravitopism of *tt4* mutants (14) by external application of flavonoids and are both consistent with a positive role of flavonoids in facilitating gravitropic curvature. *tt4* mutants lack a functional chalcone synthase and produce therefore no flavonoids, whereas adding the flavonoid precursor naringenin restores flavonoid synthesis (16, 20). However, these and our studies reached despite the different tools used opposite conclusions on the negative or positive regulatory impact of flavonoids on auxin transport. This paradox might be at least partially explained by recent work from Peer *et al.* (18) that reports opposite regulation of *PIN2* and PIN1 expression by flavonoids: whereas *PIN2* expression is enhanced in the absence of flavonoids, *PIN1* expression is depressed. Moreover, PIN1 is delocalized from the plasma membrane in the absence of flavonoids, whereas PIN2 is not (18). This suggests that flavonoids might act as negative regulators of PIN2, but as positive effectors of PIN1. The same study demonstrates that auxin reduces PIN1 expression in the root. One might speculate that this might be the underlying mechanism by which PIN1 is induced in the pin2 mutant, which owns presumably lower auxin levels in these cells that are relevant for basipetal auxin reflux. Finally, one should keep in mind that inverse modulation of root gravitropism by flavonoids may result from a combination of ABCB regulation and PIN trafficking that might be concentration-dependent and interconnected.

Although the cellular targets of flavonoid action are now known, the underlying mechanism remains elusive. Transport assays with PIN proteins suggest that flavonoids probably do not interact with PINs directly. However, flavonoids affect specific PIN expression, location, and cellular trafficking probably through interaction with regulatory proteins (18, 20). Recently, plasma membrane PIN shifts have been demonstrated to be caused by antagonistic PIN phosphorylation via protein kinase PINOID (PID) and protein phosphatase 2A (44, 45). Our data together with the fact that flavonols are routinely used as both protein phosphatase and kinase inhibitors make PID or PID-related WAG kinases (46) and/or protein phosphatase 2A-like phosphatases the most likely candidate targets for flavonol-mediated PIN-shifts.

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REFERENCES

1. Muday, G. K. (2001) *J. Plant Growth Regul.* **20,** 226–243
2. Chen, R., Hilson, P., Sedbrook, J., Rosen, E., Caspar, T., and Masson, P. H. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 15112–15117
3. Moore, I. (2002) *Curr. Biol.* **12,** R452–454
4. Vieten, A., Sauer, M., Brewer, P. B., and Friml, J. (2007) *Trends Plant Sci.* **12,** 160–168
5. Wisniewska, J., Xu, J., Seifertova, D., Brewer, P. B., Ruzicka, K., Blilou, I., Rouquie, D., Benkova, E., Scheres, B., and Friml, J. (2006) *Science* **312,** 883
6. Geisler, M., and Murphy, A. S. (2006) *FEBS Lett.* **580,** 1094–1102
7. Kerr, I. D., and Bennett, M. J. (2007) *Biochem. J.* **401,** 613–622
8. Blakeslee, J. J., Bandyopadhyay, A., Lee, O. R., Mravec, J., Titapiwatanakun, B., Sauer, M., Makam, S. N., Cheng, Y., Bouchard, R., Adamec, J., Geisler, M., Nagashima, A., Sakai, T., Martinoia, E., Friml, J., Peer, W. A., and Murphy, A. S. (2007) *Plant Cell* **19,** 131–147
9. Lewis, D. R., Miller, N. D., Splitt, B. L., Wu, G., and Spalding, E. P. (2007) *Plant Cell* **19,** 1838–1850
10. Wu, G., Lewis, D. R., and Spalding, E. P. (2007) *Plant Cell* **19,** 1826–1837
11. Swarup, R., Friml, J., Marchant, A., Ljung, K., Sandberg, G., Palme, K., and Bennett, M. (2001) *Genes Dev.* **15,** 2648–2653
12. Marchant, A., Kargul, J., May, S. T., Muller, P., Delbarre, A., Perrot-Rechenmann, C., and Bennett, M. J. (1999) *EMBO J.* **18,** 2066–2073
13. Abas, L., Benjamins, R., Malenica, N., Paciorek, T., Wisniewska, J., Moulinier-Anzola, J. C., Sieberer, T., Friml, J., and Luschnig, C. (2006) *Nat. Cell Biol.* **8,** 249–256
14. Buer, C. S., and Muday, G. K. (2004) *Plant Cell* **16,** 1191–1205
15. Buer, C. S., Muday, G. K., and Djordjevic, M. A. (2007) *Plant Physiol.* **145,** 478–490
16. Brown, D. E., Rashotte, A. M., Murphy, A. S., Normanly, J., Tague, B. W., Peer, W. A., Taiz, L., and Muday, G. K. (2001) *Plant Physiol.* **126,** 524–535
17. Murphy, A., Peer, W. A., and Taiz, L. (2000) *Planta* **211,** 315–324
18. Peer, W. A., Bandyopadhyay, A., Blakeslee, J. J., Makam, S. I., Chen, R. J., Masson, P. H., and Murphy, A. S. (2004) *Plant Cell* **16,** 1898–1911
19. Peer, W. A., and Murphy, A. S. (2006) in *The Science of Flavonoids* (Grotewold, E., ed) pp. 239–268, Springer, Berlin
20. Peer, W. A., and Murphy, A. S. (2007) *Trends Plant Sci.* **12,** 556–563
21. Taylor, L. P., and Grotewold, E. (2005) *Curr. Opin. Plant Biol.* **8,** 317–323
22. Bouchard, R., Bailly, A., Blakeslee, J. J., Oehring, S. C., Vincenzetti, V., Lee, O. R., Paponov, I., Palme, K., Mancuso, S., Murphy, A. S., Schulz, B., and Geisler, M. (2006) *J. Biol. Chem.* **281,** 30603–30612
23. Geisler, M., Blakeslee, J. J., Bouchard, R., Lee, O. R., Vincenzetti, V., Bandyopadhyay, A., Titapiwatanakun, B., Peer, W. A., Bailly, A., Richards, E. L., Ejendal, K. F. K., Smith, A. P., Baroux, C., Grossniklaus, U., Muller, A., Hrycyna, C. A., Dudler, R., Murphy, A. S., and Martinoia, E. (2005) *Plant J.* **44,** 179–194
24. Bailly, A., Sovero, V., Vincenzetti, V., Santelia, D., Bartnik, D., Koenig,

B. W., Mancuso, S., Martinoia, E., and Geisler, M. (2008) *J. Biol. Chem.* **283,** 21817–21826
25. Noh, B., Murphy, A. S., and Spalding, E. P. (2001) *Plant Cell* **13,** 2441–2454
26. Terasaka, K., Blakeslee, J. J., Titapiwatanakun, B., Peer, W. A., Bandyopadhyay, A., Makam, S. N., Lee, O. R., Richards, E. L., Murphy, A. S., Sato, F., and Yazaki, K. (2005) *Plant Cell* **17,** 2922–2939
27. Morris, M. E., and Zhang, S. (2006) *Life Sci.* **78,** 2116–2130
28. Conseil, G., Baubichon-Cortay, H., Dayan, G., Jault, J. M., Barron, D., and Di Pietro, A. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 9831–9836
29. Muller, A., Guan, C. H., Galweiler, L., Tanzler, P., Huijser, P., Marchant, A., Parry, G., Bennett, M., Wisman, E., and Palme, K. (1998) *EMBO J.* **17,** 6903–6911
30. Luschnig, C., Gaxiola, R. A., Grisafi, P., and Fink, G. R. (1998) *Genes Dev.* **12,** 2175–2187
31. Jacobs, M., and Rubery, P. H. (1988) *Science* **241,** 346–349
32. Friml, J., Benkova, E., Mayer, U., Palme, K., and Muster, G. (2003) *Plant J.* **34,** 115–124
33. Paciorek, T., Zazimalova, E., Ruthardt, N., Petrasek, J., Stierhof, Y. D., Kleine-Vehn, J., Morris, D. A., Emans, N., Jurgens, G., Geldner, N., and Friml, J. (2005) *Nature* **435,** 1251–1256
34. Utsuno, K., Shikanai, T., Yamada, Y., and Hashimoto, T. (1998) *Plant Cell Physiol.* **39,** 1111–1118
35. Rashotte, A. M., Brady, S. R., Reed, R. C., Ante, S. J., and Muday, G. K. (2000) *Plant Physiol.* **122,** 481–490
36. Peer, W. A., Brown, D. E., Tague, B. W., Muday, G. K., Taiz, L., and Murphy, A. S. (2001) *Plant Physiol.* **126,** 536–548
37. Tohge, T., Nishiyama, Y., Hirai, M. Y., Yano, M., Nakajima, J., Awazuhara, M., Inoue, E., Takahashi, H., Goodenowe, D. B., Kitayama, M., Noji, M., Yamazaki, M., and Saito, K. (2005) *Plant J.* **42,** 218–235
38. Buer, C. S., Sukumar, P., and Muday, G. K. (2006) *Plant Physiol.* **140,** 1384–1396
39. Casimiro, I., Marchant, A., Bhalerao, R. P., Beeckman, T., Dhooge, S., Swarup, R., Graham, N., Inze, D., Sandberg, G., Casero, P. J., and Bennett, M. (2001) *Plant Cell* **13,** 843–852
40. Rashotte, A. M., DeLong, A., and Muday, G. K. (2001) *Plant Cell* **13,** 1683–1697
41. Ottenschlager, I., Wolff, P., Wolverton, C., Bhalerao, R. P., Sandberg, G., Ishikawa, H., Evans, M., and Palme, K. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 2987–2991
42. Vieten, A., Vanneste, S., Wisniewska, J., Benkova, E., Benjamins, R., Beeckman, T., Luschnig, C., and Friml, J. (2005) *Development* **132,** 4521–4531
43. Blilou, I., Xu, J., Wildwater, M., Willemsen, V., Paponov, I., Friml, J., Heidstra, R., Aida, M., Palme, K., and Scheres, B. (2005) *Nature* **433,** 39–44
44. Friml, J., Yang, X., Michniewicz, M., Weijers, D., Quint, A., Tietz, O., Benjamins, R., Ouwerkerk, P. B., Ljung, K., Sandberg, G., Hooykaas, P. J., Palme, K., and Offringa, R. (2004) *Science* **306,** 862–865
45. Michniewicz, M., Zago, M. K., Abas, L., Weijers, D., Schweighofer, A., Meskiene, I., Heisler, M. G., Ohno, C., Zhang, J., Huang, F., Schwab, R., Weigel, D., Meyerowitz, E. M., Luschnig, C., Offringa, R., and Friml, J. (2007) *Cell* **130,** 1044–1056
46. Santner, A. A., and Watson, J. C. (2006) *Plant J.* **45,** 752–764
47. Kerhoas, L., Aouak, D., Cingöz, A., Routaboul, J. M., Lepiniec, L., Einhorn, J., and Birlirakis, N. (2006) *J. Agric. Food Chem.* **54,** 6603–6612
48. Routaboul, J. M., Kerhoas, L., Debeaujon, I., Pourcel, L., Caboche, M., Einhorn, J., and Lepiniec, L. (2006) *Planta* **224,** 96–107
49. Stobiecki, M., Malosse, C., Kerhoas, L., Wojlaszek, P., and Einhorn, J. (1999) *Phytochem. Anal.* **10,** 198–207
50. Morris, D. A. (2000) *Plant Growth Regul.* **32,** 161–172
51. Lomax, T. L., Muday, G. K., and Rubery, P. H. (1995) in *Plant Hormones: Physiology, Biochemistry and Molecular Biology* (Davies, P. J., ed) pp. 509–530, Kluwer, Dordrecht, Netherlands
52. Luschnig, C. (2002) *Trends Plant Sci.* **7,** 329–332

Flavonoids Redirect PIN-mediated Polar Auxin Fluxes during Root Gravitropic Responses

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Supplemental data

http://www.jbc.org/

Supplemental Figures S1, S2, S3

Supplemental Table S1, S2

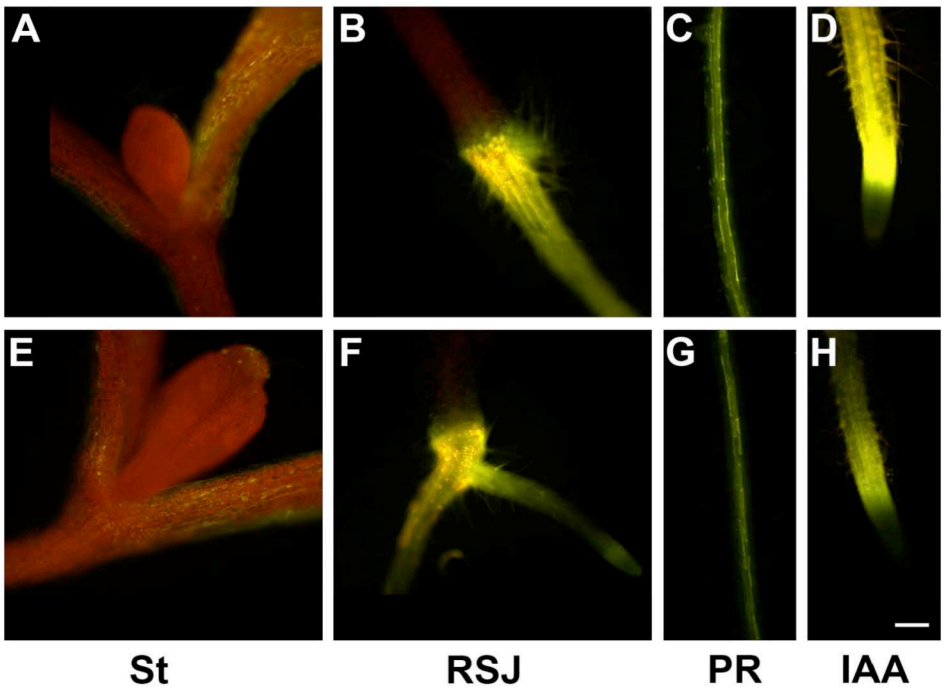


Figure S1. Flavonoid distribution in cotyledons, root-shoot junction and primary root of *pin2* mutant is not affected, but exogenous supply of IAA resulted in increased flavonoid accumulation in the EZ.

In situ flavonoid visualization of *Arabidopsis* seedlings using diphenylboric acid 2-aminoethyl ester (DPBA, a fluorescent dye that interacts with flavonoids) by epifluorescence microscopy (yellow fluorescence). These patterns were observed in all stained seedlings. **A-D**, wild type (Col Wt); **E-H**, *pin2*. St, stem; RSJ, root-shoot junction; PR, primary root; IAA, application of 100 nM IAA. Bar, 100 µm.

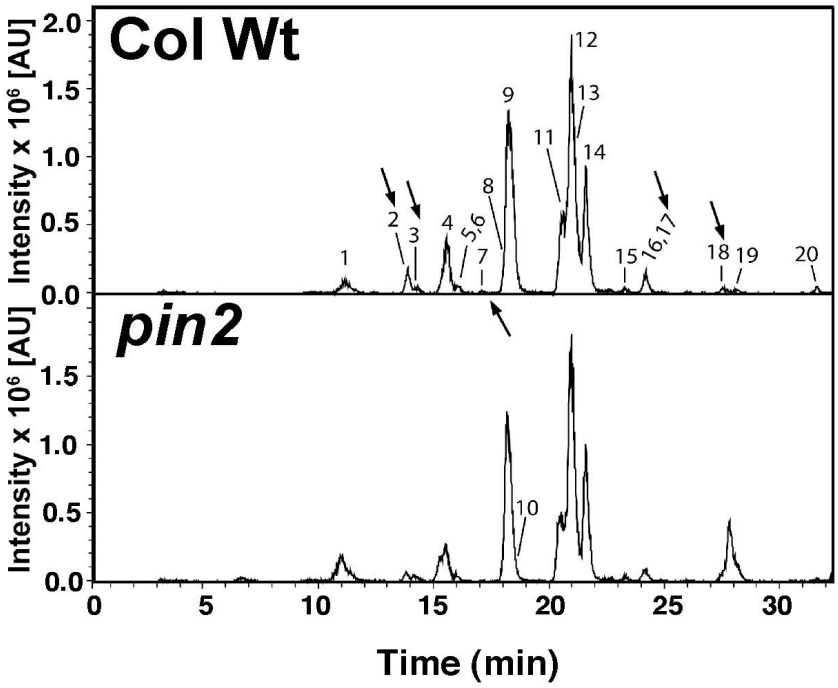


Figure S2. Accumulation of specific flavonoid glycosides in the entire *pin2* root is altered qualitatively but not quantitatively.
Representative sum of EIC of flavonoid derivatives found in wild type (Col Wt) and *pin2 (eir1-4)* entire roots analysed by LC-ESI-MS. Selected ions are *m/z* 431, 447, 463, 477, 577, 593, 609, 623, 739, and 755, 771. Altered compounds are indicated by arrows. Peak numbers correspond to flavonoid derivates listed in Table 1. Note 10-fold higher intensities at the whole root level for *pin2* compared to the root tip-elongation zone (Fig. 1G).

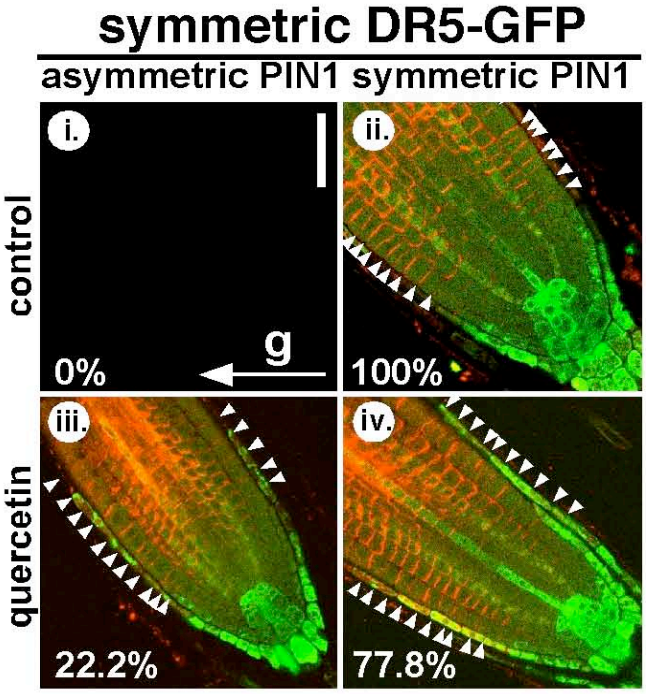


Figure S3. Flavonoid-dependent rescue of basipetal auxin reflux in *pin2* is correlated with PIN1 asymmetric distribution across gravity-stimulated tissues.
Whole-mount *in situ* immunolocalization of PIN1 protein in *pin2* after 2h of gravity stimulation. 4 dag *pin2* seedlings were transferred on media supplemented with 100nM quercetin or the solvent (control) for 24h. Red, PIN1; green, $DR5_{rev}$-GFP expression. White arrows indicate more pronounced PIN1 proteins levels at the lower or upper side of gravity stimulated root tip. Bar, 30 mm. Gravity vectors relative to the root are indicated by an arrow. Percentages indicate relative occurrence of asymmetric or symmetric PIN1 distributions with symmetric DR5-GFP signals; the total number of analysed roots showing both clear DR5-GFP and PIN1 signals was 47.

DR5-GFP 0: symm. 1: dRRC 2: pLRC 3: DEZ

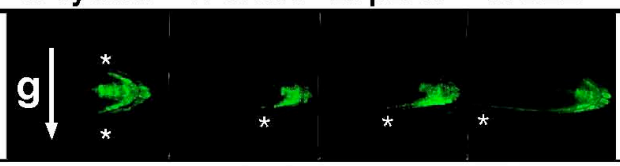
root tip orientation					total (0+1+2+3)	% (2+3) of total (0+1+2+3)
Solvent control						
A	3 17	10 3	17 1	2 0	32 21	59.4 4.8
B	4 14	15 7	9 5	0 8	28 34	32.1 38.2
C	0 4	0 2	0 4	0 0	0 10	0 40
Total	7 35	25 12	26 10	2 8	60 65	**46.7 27.7**
% of total	**11.7 53.8**	**41.7 18.5**	**43.3 15.4**	**3.3 12.3**		
Quercetin						
A	12 11	27 9	32 12	6 4	77 36	49.6 44.4
B	2 5	9 5	11 13	5 20	27 43	59.3 76.7
C	1 5	0 3	0 7	0 6	1 21	0 61.9
total	15 21	36 17	43 32	11 30	105 100	**51.4 62**
% of total	**14.3 21**	**34.3 17**	**41 32**	**10.5 30**		

Table S1: The majority of quercetin-treated *pin2* roots form IAA gradients upon gravity stimulation.

Absolute and relative numbers of wild type (black) and *pin2* (red) seedling showing IAA gradients. Definition of IAA gradients is based on the strength of IAA movement from the LRC to the EZ, as assessed by confocal imaging of DR5-GFP fluorescent signals upon 2h gravity stimulation. Gradient symmetry is quantified in arbitrary units (0= symmetric signal; 1= weak signal asymmetry, up to distal lateral root cap (dLRC); 2= intermediate signal asymmetry, up to proximal lateral root cap (pLRC); 3= strong signal asymmetry, up to elongation zone (DEZ)). Classification of root tip orientation is relative to the gravity stimulation vector, as depicted (A= neutral; B= towards the gravity stimulation vector; C= opposite to the gravity stimulation vector). Number of analysed wild type (*pin2*) seedlings was 60 (65) on solvent control and 105 (100) on 100 nM quercetin plates, respectively. Gravity vector (g) relative to the root is indicated by an arrow; symmetry of DR5-GFP signals by asterisks.

DR5-GFP 0: symm. 1: dRRC 2: pLRC 3: DEZ

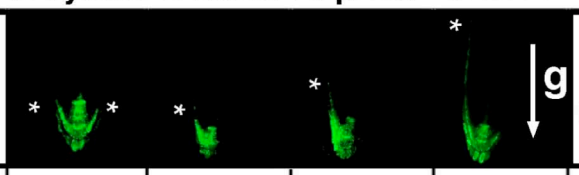
root tip orientation					total (0+1+2+3)	% (2+3) of total (0+1+2+3)
A 	51 12	5 3	0 4	0 0	56 19	0 21.1
B 	2 11	0 3	0 6	0 9	2 29	0 51.7
C 	1 10	0 4	1 0	0 1	2 15	50 6.7
total	**54 33**	**5 10**	**1 10**	**0 10**	**60 63**	**1.7 31.7**
% of total	**90 52.4**	**8.3 15.9**	**1.715.9**	**0 15.9**		

Table S2. Weak asymmetric IAA gradients were occasionally observed in vertically oriented *pin2* roots.

Absolute and relative numbers of wild type (black) and *pin2* (red) seedling showing IAA gradients. Definition of IAA gradients is based on the strength of IAA movement from the LRC to the EZ, as assessed by confocal imaging of DR5-GFP fluorescent signals upon 2h gravity stimulation. Gradient symmetry is quantified in arbitrary units (0= symmetric signal; 1= weak signal asymmetry, up to distal lateral root cap (dLRC); 2= intermediate signal asymmetry, up to proximal lateral root cap (pLRC); 3= strong signal asymmetry, up to elongation zone (DEZ)). Classification of root tip orientation is relative to the gravity stimulation vector, as depicted (A = neutral; B = to the left (lower than 90°); C = to the right (higher than 90°). Number of analysed wild type (*pin2*) seedlings was 60 (63). Gravity vector (g) relative to the root is indicated by an arrow.

5.2 Identification of an ABCB/P-glycoprotein-specific inhibitor of auxin transport by chemical genomics

Jun-Young Kim[3], Sina Henrichs[3], Valpuri Sovero, Aurélien Bailly, Stefano Mancuso, Stephan Pollmann, Daehwang Kim, Markus Geisler and Hong-Gil Nam

Abstract

Plant development and physiology is widely determined by the polar transport of the signaling molecule auxin. This process is controlled on the cellular efflux level as catalyzed by members of the PIN- (PINFORMED) and ABCB/PGP (PGLYCOPROTEIN) family that can function independently and coordinately. In this study, we have identified by means of chemical genomics a novel auxin transport inhibitor (ATI), BUM, that efficiently blocks auxin-regulated plant physiology and development. Physiological analysis and auxin transport as well as binding assays identified ABCB1, and to a lesser extend ABCB19, as key targets of BUM, while PIN proteins are apparently not affected. In some respects, BUM resembles the functionality of the diagnostic ATI, 1-*N*-naphtylphtalamic acid (NPA) but has an IC50 that is roughly a factor 30 lower. Distinct modes and targets of action compared to NPA are reflected by apically shifted root influx maxima that might be the result of altered BUM binding preferences or affinities to the ABCB nucleotide binding folds. This qualifies BUM as a valuable tool for auxin research allowing differentiating between ABCB- and PIN-mediated efflux systems. Moreover, BUM is complementary to NPA and the recently identified ATI, gravacin, by showing distinct ABCB target spectra and by lack of interference with ABCB membrane trafficking. Beside its obvious application as a powerful weed herbicide, BUM is a *bona fide* HsABCB inhibitor with the potential to restrict multidrug resistance during chemotherapy.

Own contribution

Figure 3: BUM alters auxin responses and transport *in planta*
(A) *In planta* analysis of auxin responses and transport was analyzed by confocal laser scanning microscopy for imaging of the expression pattern of the auxin-responsive reporter ProDR5::GFP upon BUM and NPA treatments in root tips of different mutants.
(C) Sample preparation for root and shoot free IAA concentrations measurements by chromatography-mass spectrometry (GC-MS) of BUM- and NPA-treated wild type seedlings.

Figure 4: Effect of BUM on PIN and ABCB abundance and locations
Confocal microscopy analysis and imaging for the protein localization of PIN1::PIN1:GFP, PIN2::PIN2:GFP, ABCB1::ABCB1:GFP and ABCB19::ABCB19:GFP fusion proteins in Arabidopsis roots upon BUM and NPA treatments.

Figure 5: BUM binds to and inhibits ABCB auxin exporters
(A) Yeast auxin export assays for IAA and BA export in *S. cerevisiae*: Test different heterologously expressed proteins in the presence of BUM and NPA.
(B) Competition Binding Assay in plant microsomes: BUM competes for NPA-binding to wild type and *pin2* microsomes.

Figure S2: BUM disrupts gravitropic root responses.
Quantification of root gravitropism of *abcb1/pgp1* and *pin2/eir1-4* alleles in comparison with corresponding wild type ecotypes in the presence and absence of BUM.

[3] Sharing co-first authorship.

Title page

Identification of an ABCB/P-glycoprotein-specific inhibitor of auxin transport by chemical genomics

Jun-Young Kim [1, *], Sina Henrichs [2, *], Valpuri Sovero [2], Aurélien Bailly [2], Stefano Mancuso [3], Stephan Pollmann [4], Daehwang Kim [5], Markus Geisler [2] and Hong-Gil Nam[1]

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Plant development and physiology is widely determined by the polar transport of the signaling molecule auxin. This process is controlled on the cellular efflux level as catalyzed by members of the PIN- (PIN-FORMED) and ABCB/PGP (P-GLYCOPROTEIN) family that can function independently and coordinately.

In this study, we have identified by means of chemical genomics a novel auxin transport inhibitor (ATI), BUM, that efficiently blocks auxin-regulated plant physiology and development. Physiological analysis and auxin transport as well as binding assays identified ABCB1, and to a lesser extend ABCB19, as key targets of BUM, while PIN proteins are apparently not affected. In some respects, BUM resembles the functionality of the diagnostic ATI, 1-*N*-naphtyl phtalamic acid (NPA) but has an IC_{50} that is roughly a factor 30 lower. Distinct modes and targets of action compared to NPA are reflected by apically shifted root influx maxima that might be the result of altered BUM binding preferences or affinities to the ABCB nucleotide binding folds. This qualifies BUM as a valuable tool for auxin research allowing differentiating between ABCB- and PIN-mediated efflux systems. Moreover, BUM is complementary to NPA and the recently identified ATI, gravacin, by showing distinct ABCB target spectra and by lack of interference with ABCB membrane trafficking. Beside its obvious application as a powerful weed herbicide, BUM is a *bona fide* HsABCB inhibitor with the potential to restrict multidrug resistance during chemotherapy.

Introduction

The auxin, IAA (indolyl-3-acetic acid) is used as clinical marker for serotonergic abnormalies (1), carcinoid testicular cancer (2) and suicidal behavior in mood disorders (3). Moreover, intravenously applied IAA is in clinical trial as cytotoxic prodrug during cancer therapy (4). In plants, IAA serves as a hormone-like signaling molecule that is a key factor in plant development and physiology (5-8). Many of its functionalities are controlled by a unique, plant specific process, the cell-to-cell or polar auxin transport (PAT) (7). However, cellular efflux is the rate-limiting step of PAT and in agreement with the chemiosmotic hypothesis, putative exporters of the PIN- (PIN-FORMED) and B subfamily of ABC transporter, ABCB/PGP/MDR (P-GLYCOPROTEIN, MULTIDRUG-RESISTANCE), families have been identified (9-11).

PIN efflux carriers show polar locations in PAT tissues, developmental, organogenetic loss-of-function phenotypes and are thought to be the determinants of a “reflux loop” in the root apex (7,12). ABCB isoforms have been identified as primary active (ATP-dependent) auxin pumps showing auxin-related, developmental (but not organogenetic) loss-of-function phenotypes (9,13,14). Despite their mostly apolar locations, they have been demonstrated to contribute to PAT and long-range auxin transport (9,15,16). Moreover, ABCB1 and ABCB19 coordinately function in basipetal reflux of auxin maxima out of the root and shoot tip (13). The immunophilin-like FKBP42, TWISTED DWARF1 (TWD1), protein was characterized as a central regulator of

ABCB-mediated auxin transport by means of protein-protein interaction (17-19). Positive regulation of ABCB1/PGP1- and ABCB19/PGP19/MDR1-mediated auxin transport accounts for overlapping phenotypes between *twd1* and *abcb1 abcb19* (15,16).
ABCB- and PIN-mediated auxin efflux can function independently and play identical cellular but separate developmental roles (14). However, ABCBs and PINs are also able to interactively and coordinately transport auxin (13). The current picture that emerges is that in interacting cells, multilaterally expressed ABCBs minimize apoplastic reflux while polar ABCB-PIN interactions provide specific vectorial auxin stream (14). However, the individual roles of ABCB-and PIN-mediated auxin flows are far from being understood.

The investigation of PAT streams was facilitated by using synthetic compounds that act as ATIs, with 1-*N*-Naphtylphtalamic acid (NPA) being the most prominent. Until today the identity, number and affinity of putative NPA-binding proteins (NBP) is still controversial (20-24). However, the current consensus is that the auxin efflux complex consists of at least two proteins: a membrane integral transporter and an NBP regulatory subunit (20-22,25). Several lines of evidence suggest that PIN proteins do not itself act as NBPs (26), therefore it was suggested that NPA blocks PAT by interfering with the cycling of auxin transporters, like PIN1 (27). However, NPA itself does not affect PIN cycling and concentrations necessary to perturb PIN cycling were largely higher than was needed for efficiently blocking PAT (23,27). Independently, ABCB1 and ABCB19 have been identified as targets of NPA (9,28,29) and high-affinity NBPs (29-31). Surprisingly, NPA was additionally shown to bind to TWD1 and NPA binding disrupted TWD1-ABCB1 interaction (15). *In planta* this leads to disruption of ABCB1 activity, suggesting that TWD1 and ABCB1 represent high- and low-affinity components of the NPA-sensitive efflux complex (15).
In the last few years, chemical genomic screens have allowed for the identification of several synthetic compounds and, in some cases respective molecular targets that interfere with auxin signalling (32,33), membrane trafficking (34-36) and auxin transport (29,37). Gravacin (3-(5-[3,4-dichlorophenyl]-2-furyl)acrylic acid) was recently identified as a strong inhibitor of root and shoot gravitropism, auxin responsiveness, and protein trafficking to the tonoplast in *Arabidopsis* (36). In a follow-up screen, inhibition of gravitropism and protein trafficking was shown to employ independent mechanics (29). Mutations in *ABCB19* confer resistance to the effect of gravacin on hypocotyl gravitropism and result in reduced binding of gravacin to microsomal fractions determining ABCB19 as the major target of gravacin (29). Consequently, gravacin was found to be a strong inhibitor of ABCB19-mediated auxin transport in *Arabidopsis* and *HeLa* cells.
In this study we screened chemical libraries of small organic compounds for plant physiological and developmental regulators and identified a novel, highly potent ATI by means of chemical genomics. A direct comparison of compound 10824 (BUM) and NPA effects on auxin-controlled plant physiology, auxin transport and drug binding reveals that BUM shares many features with NPA, like induction of pin-formed inflorescences, but binds predominantly to ABCB1. Unlike NPA and gravacin, BUM lacks growths activation at lower concentrations and does not interfere with membrane trafficking. BUM might act as a powerful tool in dissecting ABCB- and PIN-mediated auxin streams in plant physiology.

Experimental Procedures

Chemical library screens

Arabidopsis thaliana ecotype Columbia (Col) seeds were surface-sterilized, stratified at 4°C for 3 days and grown horizontally in 24 well plates (3 seeds per well; 0.5 x B5 media, 2% sucrose, 0.8% agar) at 22°C under continuous light. Seven days after germination (dag), 2 µM of organic compounds of a chemical library (containing 6.500 small organic chemicals at 50 µM in DMSO) from the Korea Chemical Bank was added manually. Plant phenotypes in respect to plant morphology, growth rate, leaf color, flowering time and senescence were monitored every 2 days by visual examination in comparison to the solvent (DMSO) control on each plate up to 14 dag.
In a secondary screen, plants were treated with various concentrations (up to 10 µM) of compounds from the Korea Chemical Bank that were structurally related to compound 10824 and thus contained the 2-(formyl)-benzoic acid core (Fig. 1B; Table S1). Phenotypes were screened for induction of pin-formed inflorescences (see Fig. 1C).

Plant material and quantification of growth

For long-term experiments, *abcb1/pgp1* (At2g36910), *abcb19/pgp19/mdr1* (At3g28860) (all ecotype Wassilewskija) and *pin2/eir1-4* (At5g57090, ecotype Columbia) were grown on 0.5 x B5 media, 2% sucrose, 0.8% phytagar under continuous light for 18 dags in sterilized plastic boxes (SPL, Korea). For all other experiments, seedlings were grown if not indicated elsewhere for 5 dag on vertical plates containing 0.5 x Murashigge and Skoog (MS) media, 1% sucrose, 1% phytagar in the dark or at 16h light per day. For growth quantification, seedlings were transferred on drug containing plates (0-50 µM). After 5, 7, 9 and 11 dag seedlings were aligned on 1% phytoagar media, images were scanned and root and hypocotyl lengths and lateral root numbers (7, 9 and 11 dag) were measured using Scion Image software (Scion Corporation, Frederick, MD). For determination of IC_{50} values, root lengths of 7 dag seedlings on 0-80 µM NPA and 0-20 µM BUM was quantified and IC_{50} values were calculated using sigmoidal dose-response fits. All experiments were performed at minimum as triplicates with 20-30 seedlings per each experiment.

***In planta* analysis of auxin responses and transport**

Homozygous F4 generations of *A. thaliana* wild-type, *pin2/eir1-4*, *abcb1/pgp1*, *abcb19/pgp19/mdr1*, mutants expressing the maximal auxin-inducible reporter Pro_{DR5}:GFP (41) were grown vertically for 5 dags and analyzed by confocal laser scanning microscopy (Leica, DMIRE2). In some cases, seedlings were transferred for an additional 12 h onto new plates containing 0.5 µM BUM, 5 µM NPA or the solvent DMSO. For histological signal localization, differential interference contrast (DIC) and GFP images were merged electronically using Photoshop 7.0 (Adobe Systems).

A platinum microelectrode was used to monitor IAA fluxes in *Arabidopsis* roots as described previously (20, 42). For measurements, plants were grown in hydroponic cultures and used at 5 days after germination. Differential current from an IAA-selective microelectrode was recorded in the absence and presence of 5 µM NPA, BUM or gravacin (29).

Endogenous free IAA was quantified from shoot and root segments of MeOH extracted seedlings by using gas chromatography-mass spectrometry (GC-MS) as described in Bouchard et al. (2006). Seedlings were analyzed after 24h treatments with 5 µM NPA or 0.5 µM BUM. Data are means of three independent lots of 30–50 seedlings each.

Yeast auxin transport assays

IAA transport experiments were performed as in (15). In short, *S. cerevisiae* strains JK93d expressing PIN2-HA or ABCB1 from pADE-PIN2-HA (38) or pNEV-PGP1 (9) were loaded with [^{3}H]-IAA (20 Ci/mmol; American Radiolabeled Chemicals Inc.) and [^{14}C]-benzoic acid (BA; (58 mCi/mmol; American Radiolabeled Chemicals Inc.) and retained radioactivity was quantified after 0 and 10 min incubation at 30°C. ABCB1- and PIN2-mediated IAA and BA export is expressed as relative retention of initial (maximal) loading (t = 0 min), which is set to 100%. Presented are average values from 6–8 independent experiments.

NPA binding studies

NPA binding assays using *Arabidopsis* microsomes were performed as described elsewhere (ABA). Four replicates of each 20 µg of protein were incubated with 10 nM [^{3}H]NPA (60 Ci/mmol) and 10 nM [^{14}C]BA (58 mCi/mmol) in the presence and absence of 10 µM NPA. For competition experiments, 100 nM BUM was added. Reported values are the means of specific binding ([^{3}H]NPA bound in the absence of cold NPA (total) minus [^{3}H]NPA bound in the presence of cold NPA (unspecific)) from three independent experiments with four replicates each.

BRET analysis

Microsomes from yeast JK93d expressing PGP1-YFP and TWD1-rLuc (15) were prepared in the presence or absence of 5 µM NPA, BUM or gravacin or adequate amounts of solvents. BRET signals were recorded in the presence of 5 µM coelenterazine (Biotium Inc.) and BRET ratios were calculated as described (15). The results are the average of 10 readings collected every minute; presented are average values from 6–10 independent experiments with four replicas.

Data Analysis

Data were analyzed using Prism 4.0b (GraphPad Software, San Diego, CA). AtABCB1 structure modeling was performed using PyMol 0.99 (www.pymol.org) and maxium-entropy based ligand binding was computed using MEDock (http://medock.csie.ntu.edu.tw/) as described in

Yang et al. (2009). Drug docking was confirmed by using ZDOCK (http://zdock.bu.edu). Drug 3D structures were energy minimized using PRODRG2 http://davapc1.bioch.dundee.ac.uk/prodrg/index.htm) and solely polar hydrogens are displayed (usual atom color code).

Results

A chemical library screen for growth and developmental regulators

To identify growth and developmental regulators, we screened *A. thaliana* (ecotype Columbia; Col Wt) seedlings with a chemical library (Korean Chemical Bank, KRICT, Korea) composed of 6,500 small organic compounds. Seeds were germinated in 24-well plates, and 7 days after germination (dag), 2 µM of a different library compound was added to each well. Plant phenotypes in respect to plant morphology, growth rate, leaf color, flowering time and senescence were monitored every 2 days by visual examination in comparison to solvent (DMSO) controls. Among the various chemical compounds that led to altered plant morphology, compound number 10824 (2-[4-(diethylamino)-2-hydroxybenzoyl]benzoic acid) produced a drastic phenotype, including dark green, epinastic leaves (Fig. 1A), suppression of primary and secondary roots (Fig. 1D) and abnormal, pin-formed inflorescences (Fig. 1C). We narrowed our focus to this compound 10824, subsequently named BUM after the Korean football player Cha Bum-Kun, verifying consistently growth inhibition under a variety of assay conditions in follow-up screenings.

The fact that BUM/10824 produced pin-formed inflorescences in analogy to the well established auxin transport inhibitor (ATI), NPA 2-(1-naphthylcarbamoyl)benzoic acid or 1-N-Naphtyl phtalamic acid) (22,23,39), and that both contain a 2-(formyl)-benzoic acid core (Fig. 1B), prompted us to compare growth defects between BUM and NPA over a wide concentration range. BUM induces pin-shaped inflorescences and reduces primary root growth at 0.5 µM, which is roughly 20-times lower than what is needed for NPA (Fig. 1C and D).

In a secondary screen, we tested compounds that contained a 2-(formyl)-benzoic acid core taken from the Korea Chemical Bank and Chembridge chemical libraries. None of the six tested compounds A-F was able to induce pin-shaped inflorescences over a wide concentration range up to 10 µM (Fig. S1), suggesting that not the 2-(formyl)-benzoic acid core alone but side chains determine functionality.

BUM affects auxin-controlled plant growth

These findings suggested that BUM influences plant growth and development in analogy to the ATI, NPA, but has a stronger effect. Therefore, we quantified root- and hypocotyl lengths, known to be inversely controlled by auxin, in the presence of BUM and NPA in more detail. BUM drastically reduced primary root growth of light-grown wild type seedlings (Fig. 2A and B) with an apparent IC_{50} of 0.4 µM (Fig. S5) which is roughly a factor 30 less than what is needed with NPA (IC_{50} = 12.8 µM). A similar effect was found also for hypocotyl elongation of light-grown seedlings. NPA shows, in agreement with previous reports on roots (40), a stimulating and inhibitory result on hypocotyl elongation under light at nM and µM concentrations, respectively. Such a biphasic behavior was not found for BUM in the used concentration range (0 - 20 µM) suggesting, despite widely overlapping effects, a different mode of action or targets (Fig. 2).

A shoot-derived auxin pulse known to be efficiently inhibited by NPA (41-43) tightly controls lateral root emergence. Not unexpectedly, 0.1 µM BUM drastically blocked LR formation in both of the tested *Arabidopsis* ecotypes, Col and Ws, by roughly 50%; enhanced sensitivities compared to 0.5 µM NPA (roughly 25% inhibition) were in-line with what was found for primary root growth. Next we tested root gravitropism, another hallmark of auxin-controlled plant physiology (44,45). BUM disrupted root bending in wild type seedlings drastically (Fig. S2); interestingly, the ecotype Col revealed higher sensitivities (77% inhibition) compared to the ecotype Ws (58% inhibition) not found for NPA (15,40). Similarly to LR formation, 0.1 µM of BUM (Fig. S2) was more efficient than 5 µM NPA assayed in both ecotypes in parallel (not shown; (15,40)).

Interestingly, root and hypocotyl growth inhibition by BUM and NPA was light-dependent and less pronounced in dark-grown, etiolated seedlings (Fig. S4). Moreover, shoot hook formation and opening of etiolated seedlings was inhibited by 5 µM BUM but not by NPA (Fig. 2A), that requires higher concentrations as was shown before (46-48). This is in agreement with the concept that auxin has a more important role in elongation and bending responses in light- than in dark-grown seedlings (47,48).

These results provide evidence that BUM, like

NPA, blocks many aspects of plant physiology that are controlled by the polar transport of auxin.

BUM alters the polar transport of auxin

To test our conclusions derived from growth experiments and to substantiate the physiological relevance of the proposed BUM function *in planta*, we investigated BUM sensitivity of wild type roots in comparison with NPA using three different approaches.

First, analysis of the auxin-responsive reporter construct Pro_{DR5}-GFP (49) revealed that in analogy to NPA (15), BUM disrupted basipetal, root-to-shoot auxin reflux and enhanced the DR5-GFP signal in the quiescent center (QC), columella initials (CIn), and S1 cells but reduced signals in columella S2, S3 and cap cells (CC) (Fig. 3A). BUM inhibition, although used at 10-fold lower concentration, was more drastic compared to NPA, resulting in enhanced DR5-GFP signal extending the quiescent center, columella initials and S1 cells into initials of epidermis, endodermis and stele upon BUM treatment (asterisks) compared to NPA. As shown before, this auxin block was more pronounced in the Col ecotype than in the Ws ecotype (15).

Second, we employed an IAA-specific microelectrode that has become a reliable tool for non-invasively recording IAA influxes into the root transition zone (15,16,50,51). IAA influx in this zone is characterized by a distinct peak at 200 µm from the root tip and is consistent with the current auxin reflux model (12) and a measure for PAT. In agreement with DR5-GFP imaging, IAA influx peaks were strongly and similarly reduced by 5 µM BUM and NPA (Fig. 3B). The magnitude of inhibition caused by BUM and NPA phenocopies genetical reductions of influx peaks found for *ABCB1/ABCB19* auxin transporter loss-of-function roots (15). However, gravacin, a recently identified inhibitor of gravitropism (29), had a less pronounced inhibitory effect on IAA influx as with *abcb1* or *abcb19* single mutant roots (15). This is in good agreement with the reported concept that gravacin binds to and inhibits primarily ABCB19 and not ABCB1 (29). Interestingly, BUM resulted in an additional shift of the influx maximum ca. 40 nm in the apical direction not found with NPA or gravacin that might account for more drastic growth inhibition despite similar reduction of influx peaks.

Third, we analyzed free auxin (IAA) levels in vertically grown root and shoot portions of 5 dag (days after germination) wild type seedlings treated with BUM and NPA. While 5 µM NPA had only a minor effect on auxin root-shoot ratios, 0.5 µM BUM significantly enhanced both root and shoot auxin levels (Fig. 3C), suggesting a block of basipetal delivery of IAA from the shoot to the root and *vice versa*. Elevated auxin levels are in agreement with and explain reduced root lengths caused by BUM. Effects of NPA and BUM treatments were not additive (Fig. 3C) indicating overlapping modes of action and/or targets. In summary these data support the concept that BUM in analogy to NPA blocks PAT.

BUM modifies PIN but not ABCB1 expression

Inhibition of PAT-driven plant growth and gravitropism can be achieved via two pathways, by blocking the trafficking (27,28) and the direct or indirect inhibition of auxin transporters. Accordingly, gravacin was identified in a chemical genomics screen for gravitropic modulators and shown to block trafficking of the vacuolar marker GFP-∂TIP and ABCB19, but also to bind to and inhibit ABCB19 (29). Therefore, we questioned whether BUM would interfere with the abundance and location of the major players in basipetal auxin transport, ABCB1 and ABCB19 on one hand, and PIN1 and PIN2 on the other. While BUM (like NPA) had only mild effects on the expression (ABCB1 was slightly up-regulated in the stele) and no significant effect on the location of ABCB1- and ABCB19-GFP fusion, NPA, and more pronouncedly BUM, enhanced PIN1- and lowered PIN2-GFP signals in the stele and epidermal/cortical cell files, respectively (Fig. 4). Unchanged expression of ABCB proteins and reduced PIN2 abundance upon BUM and NPA addition is supported by semi-quantitative RT-PCR analysis (Fig. S3) and for NPA as well by gene chip analysis (www.genevestigator.ethz.ch).

Interestingly, NPA, and again more strongly BUM, induced ectopic PIN1-GFP expression in PIN2 locations (epidermis and cortex; inset Fig. 4), as described previously for auxin transport modulator quercetin (40,52). In light of these findings, up- and down-regulation of PIN proteins in their non-native environments might be of indirect nature and triggered by elevated auxin levels in these tissues caused by the blocking of PAT. Inverse impact of IAA on PIN1- and PIN2 expression was reported recently (40,52,53). In summary, BUM has, unlike gravacin, only minor impact on ABCB expression and abundance but it, like NPA, indirectly interferes with PIN expression probably via altered IAA levels.

ABCBs are the primary targets of BUM and

NPA

The above suggested that auxin exporters, the primary control units of PAT, might be the direct targets of BUM action. However, the current picture is that ABCBs and the interacting ABCB1,19-regulator, TWD1/FKBP42, but not PIN proteins represent predicted low- and high affinities, respectively, NPA binding proteins (15). This is supported by recent studies demonstrating ABCB1 and ABCB19 to bind NPA resulting in inhibition of efflux activity (9,15,29), while PIN1 did not seem to bind NPA (29).

Our data showing that lateral root formation in *abcb1* but not in *pin2* roots is BUM insensitive (Fig. 2C) are therefore in agreement. Moreover, IAA export analysis in yeast clearly demonstrated that ABCB1 but not PIN2 is inhibited significantly by BUM and by NPA although to a lesser extent. Inhibition is specific, as background activities monitored simultaneously by the non-ABCB1 substrate benzoic acid is not affected (Fig. 5A). Finally, NPA binding studies using *Arabidopsis* microsomes support the yeast data by demonstrating that ABCB1, but again not PIN2 functions, as an NPA-binding protein (Fig. 5B). Interestingly, ABCB19 contributes less to NPA binding compared to ABCB1, which is in contrast to previous data that determined ABCB19 as primary NBP (29). However, the previous study employed different starting material and microsomal preparations for the binding studies that might influence individual ABCB abundance (29). Importantly, BUM significantly competes for NPA binding on both wild type ecotypes and *pin2* microsomes but not on *abcb1* and to a minor degree on *abcb19* membranes. Enhanced NPA binding caused by BUM competition on *abcb1* membranes might be indirect as the same was found with the unspecific control, benzoic acid, assayed in parallel (not shown).

Recently, single loss-of-function roots, *pin2*, *abcb1* or *abcb19*, were shown to be NPA sensitive using gravitropism assays (15,40). In agreement, DR5-GFP imaging (Fig. 3) revealed no dramatic differences between *pin2* and *abcb1* roots in comparison with corresponding wild types. However, based on gravitropism assays (Fig. S2) and in contrast to what was recently found for NPA (15,40), *abcb1* roots were significantly less affected by BUM: 35.3% (percent occurrence of 60, 90 and 120° bending between inhibitor and solvent control (see Materials and Methods)) of *abcb1* roots bended efficiently on BUM but only 26.8% of the corresponding wild type. Even higher resistance was found for *pin2* root gravitropism (Fig. S2); however, similar inhibition by BUM in the wild type suggests that this is mainly due to the strong genetic effect of the *pin2* mutation. Only partial resistance found for *abcb1* toward BUM is probably caused by partial functional redundancy between ABCB1 and ABCB19 that was recently confirmed by the finding that *abcb1 abcb19* and *twd1*, roots own reduced sensitivities toward NPA (15).

In summary, quantification of physiological parameters, drug binding and transport data indicate that ABCBs, probably primarily ABCB1, but not PIN proteins are direct BUM targets.

In order to understand functional differences between BUM and NPA inhibitor activities, we computed BUM and NPA docking to the *Arabidopsis* ABCB1 structure that was modeled on the crystal structure of ABCB-related multidrug efflux pump Sav1866 (54,55). NPA docks to both nucleotide binding domains (NBDs) interestingly, primarily to grooves between coupling helices and Q loops (Fig. 5C), the main mechanics connecting NBDs and transmembrane domains (TMDs) (55,56). In contrast, BUM only docks to the corresponding pocket of NBD2 and additionally to an NBD1-NBD2 interface. Interestingly, BUM additionally has a high affinity (around -50 kcal/mol) to a second NBD1-NBD2 interface where no NPA binding was predicted; this might account for its severe inhibition.

NPA was recently demonstrated to block ABCB-mediated auxin export by disrupting TWD1-ABCB1 interaction (9,15). Not surprisingly, BUM also disrupted TWD1-ABCB1 interaction as monitored by established yeast BRET assays (15). The fact that, based on docking studies, BUM probably binds like NPA to the ABCB1 NBD2 is inline with experimental data (9,15,16) and recent modeling of TWD1-ABC interfaces (57). More severe disruption, probably caused by BUM binding to NBD1-NBD2 interfaces, suggests that BUM achieves TWD1-ABCB1 disruption either by long-range, intramolecular movements proposed for ABCBs (54,56) or by binding to TWD1 in analogy to NPA (15). Although *twd1* is gravacin-insensitive (29), gravacin does not disrupt TWD1-ABCB1 interaction. Therefore, TWD1 is apparently not a direct target of gravacin as has been proposed recently (29).

Discussion

In this study, we have identified by means of chemical genomics a novel ATI that efficiently

blocks auxin-related plant physiology and development. Recent analyses of PIN and ABCB transport mechanisms suggest independent (or sometimes even opposite) and at certain domains additive and synergistic actions (13,14). Based on our findings, the key targets of BUM (and NPA) are ABCBs, while PINs are apparently less affected which is in agreement with previous findings (21,28,29). This makes BUM a valuable tool for auxin research allowing differentiation between ABCB- and PIN-mediated efflux systems. On the other hand, our work also suggests that pin-formed inflorescences caused by BUM and NPA and that phenocopy *PIN1* loss-of-function mutations, are primarily caused by ABCB transport inhibition of the functional ABCB-PIN efflux complex.

Our findings also demonstrate that ABCBs, the cellular targets of BUM- and NPA-induced inhibition in *Arabidopsis*, are part of a light-controlled developmental pathway which is in agreement with the concept that auxin has a more important role in elongation and bending responses in the light (47,48). This concept was recently genetically supported by demonstrating that the photoreceptors, phytochromes and cryptochromes, regulate differential growth of *Arabidopsis* hypocotyls in an ABCB-dependent manner (48)

In many physiological respects and partially also structurally, BUM resembles NPA functionality but does not show activation of plant growth at lower concentrations. BUM acts roughly a factor 20 more strongly than NPA, which seems to be mainly caused by apically shifted root influx maxima. This again might be the result of altered binding preferences or affinities to the ABCB NBDs. However, based on our binding studies, the primary BUM target seems to be ABCB1. This makes BUM complementary to NPA that has been shown to affect beside ABCB19 also other ABCBs (9,28,58). BUM apparently also acts differently compared to the recently identified ATI, gravacin, which primarily inhibits ABCB19 (29). However, as shown by BRET analysis, BUM does, unlike gravacin, also alter TWD1 function, suggesting that BUM might indirectly also regulate ABCB19 activity by disrupting TWD1-ABCB19 interaction. Another advantage is that BUM, unlike gravacin, apparently does not interfere with ABCB trafficking.

Beside its academic usage as an ATI and its obvious potential as powerful weed herbicide, BUM might have a direct clinical impact, as multidrug resistance toward many anti-cancer drugs is largely caused by HsABCB1, leading often to chemotherapy ineffectiveness. Interestingly, in contrast to substrate specificities (55), human and plant ABCBs share broad inhibitor sensitivities, which was demonstrated for the flavonol quercetin that acts both as modulator of auxin transport and as inhibitor of mammalian and plant ABCBs, and for clinically relevant ABCB inhibitors, like cyclosporine A and verapamil (9,16). Based on our findings, plant ABCB inhibitors, such as BUM and NPA, are therefore *bona fide* HsABCB inhibitors that might suppress MDR when co-administered with anti-cancer drugs.

References

1. D'Andrea, G., Granella, F., Alecci, M., and Manzoni, G. C. (1998) *Cephalalgia* **18**, 94-96
2. Stroosma, O. B., and Delaere, K. P. (2008) *BJU international* **101**, 1101-1105
3. Mann, J. J., and Currier, D. (2007) *Arch Suicide Res* **11**, 3-16
4. Folkes, L. K., and Wardman, P. (2001) *Biochem Pharmacol* **61**, 129-136
5. Blakeslee, J. J., Peer, W. A., and Murphy, A. S. (2005) *Curr Opin Plant Biol* **8**, 494-500
6. Kepinski, S., and Leyser, O. (2005) *Curr Biol* **15**, R208-210
7. Vieten, A., Sauer, M., Brewer, P. B., and Friml, J. (2007) *Trends Plant Sci*
8. Benjamins, R., and Scheres, B. (2008) *Annu Rev Plant Biol* **59**, 443-465
9. Geisler, M., Blakeslee, J. J., Bouchard, R., Lee, O. R., Vincenzetti, V., Bandyopadhyay, A., Titapiwatanakun, B., Peer, W. A., Bailly, A., Richards, E. L., Ejendal, K. F., Smith, A. P., Baroux, C., Grossniklaus, U., Muller, A., Hrycyna, C. A., Dudler, R., Murphy, A. S., and Martinoia, E. (2005) *Plant J* **44**, 179-194
10. Geisler, M., and Murphy, A. S. (2006) *FEBS Lett* **580**, 1094-1102
11. Petrasek, J., Mravec, J., Bouchard, R., Blakeslee, J. J., Abas, M., Seifertova, D., Wisniewska, J., Tadele, Z., Kubes, M., Covanova, M., Dhonukshe, P., Skupa, P., Benkova, E., Perry, L., Krecek, P., Lee, O. R., Fink, G. R., Geisler, M., Murphy, A. S., Luschnig, C., Zazimalova, E., and Friml, J. (2006) *Science* **312**, 914-918
12. Blilou, I., Xu, J., Wildwater, M., Willemsen, V., Paponov, I., Friml, J., Heidstra, R., Aida, M., Palme, K., and Scheres, B. (2005) *Nature* **433**, 39-44
13. Blakeslee, J. J., Bandyopadhyay, A., Lee, O. R., Mravec, J., Titapiwatanakun, B., Sauer, M., Makam, S. N., Cheng, Y., Bouchard, R., Adamec, J., Geisler, M., Nagashima, A., Sakai, T., Martinoia, E., Friml, J., Peer, W. A., and Murphy, A. S. (2007) *Plant Cell* **19**, 131-147
14. Mravec, J., Kubes, M., Bielach, A., Gaykova, V., Petrasek, J., Skupa, P., Chand, S., Benkova, E., Zazimalova, E., and Friml, J. (2008) *Development* **135**, 3345-3354
15. Bailly, A., Sovero, V., Vincenzetti, V., Santelia, D., Bartnik, D., Koenig, B. W., Mancuso, S., Martinoia, E., and Geisler, M. (2008) *J. Biol. Chem.* **283**, 21817-21826
16. Bouchard, R., Bailly, A., Blakeslee, J. J., Oehring, S. C., Vincenzetti, V., Lee, O. R., Paponov, I., Palme, K., Mancuso, S., Murphy, A. S., Schulz, B., and Geisler, M. (2006) *J Biol Chem* **281**, 30603-30612
17. Abas, L., Benjamins, R., Malenica, N., Paciorek, T., Wisniewska, J., Moulinier-Anzola, J. C., Sieberer, T., Friml, J., and Luschnig, C. (2006) *Nat Cell Biol* **8**, 249-256
18. Geisler, M., and Bailly, A. (2008) *Trends Plant Sci*
19. Geisler, M., Kolukisaoglu, H. U., Bouchard, R., Billion, K., Berger, J., Saal, B., Frangne, N., Koncz-Kalman, Z., Koncz, C., Dudler, R., Blakeslee, J. J., Murphy, A. S., Martinoia, E., and Schulz, B. (2003) *Mol Biol Cell* **14**, 4238-4249
20. Cox, D. N., and Muday, G. K. (1994) *Plant Cell* **6**, 1941-1953
21. Luschnig, C. (2001) *Curr Biol* **11**, R831-833
22. Michalke, W., Gerard, F. K., and Art, E. G. (1992) *Planta* **187**, 254-260
23. Petrasek, J., Cerna, A., Schwarzerova, K., Elckner, M., Morris, D. A., and Zazimalova, E. (2003) *Plant Physiol* **131**, 254-263
24. Sussman, M. R., and Gardner, G. (1980) *Plant Physiol* **66**, 1074-1078
25. Bernasconi, P. (1996) *Physiologia Plantarum* **96**, 205-210
26. Lomax, T. L., Muday, G. K., and Rubery, P. H. (1995) Auxin transport. in *Plant Hormones: Physiology, Biochemistry and Molecular Biology* (Davies, P. J. ed., Kluwer, Dordrecht, Netherlands
27. Geldner, N., Friml, J., Stierhof, Y. D., Jurgens, G., and Palme, K. (2001) *Nature* **413**, 425-428
28. Nagashima, A., Uehara, Y., and Sakai, T. (2008) *Plant Cell Physiol* **49**, 1250-1255
29. Rojas-Pierce, M., Titapiwatanakun, B., Sohn, E. J., Fang, F., Larive, C. K., Blakeslee, J., Cheng, Y., Cutler, S. R., Peer, W. A., Murphy, A. S., and Raikhel, N. V. (2007) *Chem Biol* **14**, 1366-1376
30. Murphy, A. S., Hoogner, K. R., Peer, W. A., and Taiz, L. (2002) *Plant Physiol* **128**, 935-950
31. Noh, B., Murphy, A. S., and Spalding, E. P. (2001) *Plant Cell* **13**, 2441-2454
32. Armstrong, J. I., Yuan, S., Dale, J. M., Tanner, V. N., and Theologis, A. (2004) *Proc Natl Acad Sci U S A* **101**, 14978-14983
33. Zhao, Y., Dai, X., Blackwell, H. E., Schreiber, S. L., and Chory, J. (2003) *Science* **301**, 1107-1110

34. Norambuena, L., Hicks, G. R., and Raikhel, N. V. (2009) *Methods in molecular biology (Clifton, N.J* **495**, 133-143
35. Robert, S., Chary, S. N., Drakakaki, G., Li, S., Yang, Z., Raikhel, N. V., and Hicks, G. R. (2008) *Proc Natl Acad Sci U S A* **105**, 8464-8469
36. Surpin, M., Rojas-Pierce, M., Carter, C., Hicks, G. R., Vasquez, J., and Raikhel, N. V. (2005) *Proc Natl Acad Sci U S A* **102**, 4902-4907
37. Savaldi-Goldstein, S., Baiga, T. J., Pojer, F., Dabi, T., Butterfield, C., Parry, G., Santner, A., Dharmasiri, N., Tao, Y., Estelle, M., Noel, J. P., and Chory, J. (2008) *Proc Natl Acad Sci U S A* **105**, 15190-15195
38. Luschnig, C., Gaxiola, R. A., Grisafi, P., and Fink, G. R. (1998) *Genes Dev* **12**, 2175-2187
39. Morris, D. A. (2000) *Plant Growth Regul* **32**, 161-172
40. Santelia, D., Henrichs, S., Vincenzetti, V., Sauer, M., Bigler, L., Klein, M., Bailly, A., Lee, Y., Friml, J., Geisler, M., and Martinoia, E. (2008) *J Biol Chem* **283**, 31218-31226
41. Benkova, E., Michniewicz, M., Sauer, M., Teichmann, T., Seifertova, D., Jurgens, G., and Friml, J. (2003) *Cell* **115**, 591-602
42. Bhalerao, R. P., Eklof, J., Ljung, K., Marchant, A., Bennett, M., and Sandberg, G. (2002) *Plant Journal* **29**, 325-332
43. Casimiro, I., Beeckman, T., Graham, N., Bhalerao, R., Zhang, H. M., Casero, P., Sandberg, G., and Bennett, M. J. (2003) *Trends in Plant Science* **8**, 165-171
44. Rashotte, A. M., Brady, S. R., Reed, R. C., Ante, S. J., and Muday, G. K. (2000) *Plant Physiol* **122**, 481-490
45. Swarup, R., Kramer, E. M., Perry, P., Knox, K., Leyser, H. M., Haseloff, J., Beemster, G. T., Bhalerao, R., and Bennett, M. J. (2005) *Nat Cell Biol* **7**, 1057-1065
46. Achard, P., Vriezen, W. H., Van Der Straeten, D., and Harberd, N. P. (2003) *Plant Cell* **15**, 2816-2825
47. Jensen, P. J., Hangarter, R. P., and Estelle, M. (1998) *Plant Physiol* **116**, 455-462
48. Nagashima, A., Suzuki, G., Uehara, Y., Saji, K., Furukawa, T., Koshiba, T., Sekimoto, M., Fujioka, S., Kuroha, T., Kojima, M., Sakakibara, H., Fujisawa, N., Okada, K., and Sakai, T. (2008) *Plant J* **53**, 516-529
49. Ottenschlager, I., Wolff, P., Wolverton, C., Bhalerao, R. P., Sandberg, G., Ishikawa, H., Evans, M., and Palme, K. (2003) *Proc Natl Acad Sci U S A* **100**, 2987-2991
50. Mancuso, S., Marras, A. M., Magnus, V., and Baluska, F. (2005) *Anal Biochem* **341**, 344-351
51. Santelia, D., Vincenzetti, V., Azzarello, E., Bovet, L., Fukao, Y., Duchtig, P., Mancuso, S., Martinoia, E., and Geisler, M. (2005) *FEBS Lett* **579**, 5399-5406
52. Peer, W. A., Bandyopadhyay, A., Blakeslee, J. J., Makam, S. N., Chen, R. J., Masson, P. H., and Murphy, A. S. (2004) *Plant Cell* **16**, 1898-1911
53. Vieten, A., Vanneste, S., Wisniewska, J., Benkova, E., Benjamins, R., Beeckman, T., Luschnig, C., and Friml, J. (2005) *Development* **132**, 4521-4531
54. Dawson, R. J., and Locher, K. P. (2006) *Nature* **443**, 180-185
55. Yang, Y., Bailly, A., Geisler, M., Martinoia, E., and Murphy, A. (2009) submitted
56. Locher, K. P. (2008) *Philos Trans R Soc Lond B Biol Sci*
57. Granzin, J., Eckhoff, A., and Weiergraber, O. H. (2006) *J Mol Biol* **364**, 799-809
58. Terasaka, K., Blakeslee, J. J., Titapiwatanakun, B., Peer, W. A., Bandyopadhyay, A., Makam, S. N., Lee, O. R., Richards, E. L., Murphy, A. S., Sato, F., and Yazaki, K. (2005) *Plant Cell* **17**, 2922-2939

Footnotes

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Author contributions

H.G.N. and M.G. designed the research. Y.K. performed chemical genomics screens, RT-PCR and plant growth analysis; S.H. performed auxin transport, DR5-GFP and root gravitropism, PIN1,2-GFP and ABCB1,19-GFP and drug binding analyses; S.P. analyzed free auxins; V.S. quantified TWD1-ABCB1 interaction; D.K. made chemical pools donated to the Korea Chemical Bank library; A.B. performed

structure modeling and inhibitor docking; M.G. wrote the manuscript.

Abbreviations
IAA, indole-3-acetic acid; PAT, polar auxin transport; ABCB, ATP-binding cassette protein subfamily B; PIN, pin-formed; MDR, multidrug resistance; PGP, phosphoglycoprotein, TMD, transmembrane domain; ICL, intracytoplasmic loops; NBD, nucleotide-binding domain; dag, days after germination

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Figure Legends

Fig. 1: A Chemical genomic screen identified a novel auxin transport inhibitor-like compound inducing pin-formed inflorescences.
(A) A microtiter-based screening strategy using a chemical library from the Korean Chemical Bank identified compound 10824 (BUM) as a strong modifier of plant development.
(B) 3D structures of BUM in comparison with established auxin transport inhibitor, NPA.
(C) BUM induced pin-formed inflorescences. Note, that BUM concentrations (1 µM; C) necessary for pin-formed inflorescence induction are roughly 20-fold lower compared to NPA (10 µM; C, inset).
(D) BUM reduces strongly primary root growth, which is not found with NPA. Scale bars are 2 cm.

Fig. 2: BUM reduces root and hypocotyl growth in the light.
(A) Phenotype of BUM and NPA (each 0.5 µM) treated light- (upper panel) and dark-grown (lower panel) seedlings 5 dag (days after . Note that seedlings grown in the presence of BUM are hook-less (white arrow). Scale bar is 1 cm.
(B) Dose-dependency of BUM and NPA treatments on primary root and hypocotyls lengths; absolute root and hypocotyl lengths were 29.5 ± 4.4 mm and 2.7 ± 0.5 mm, respectively.
(C) Reduction of lateral root numbers caused by BUM (0.1 µM) and NPA (0.5 µM) treatments 11 dags. Note that *abcb1* in contrast to wild-type and *pin2* is less sensitive to BUM.
Data are mean ± SD (n = 3 with each 20-30 seedlings); significant differences (unpaired *t* test with Welch's correction, $p < 0.05$) between inhibitor and solvent treatments are indicated by asterisks.

Fig. 3: BUM alters auxin responses and transport *in planta*
(A) Expression of the auxin-responsive reporter ProDR5-GFP (green) upon BUM (0.5 µM) and NPA (5 µM) treatments (24h) in root tips. BUM and NPA enhance DR5-GFP signals in the quiescent center (QC), columella initials (CIn), and S1 cells but reduce signals in columella S2, S3 and cap cells (CC); S2 and S3 borders are marked with arrowheads. Note stronger extensions of GFP signals from quiescent center, columella initials and S1 cells into initials of epidermis, endodermis and stele upon BUM treatment (asterisks) compared to NPA. Scale bar is 200 µm.
(B) IAA influx profile along wild type roots in the presence of inhibitors (5 µM) measured using an IAA-specific microelectrode; positive fluxes represent a net IAA influx. Data are means ± S.E. (n = 12). Note that BUM results in a reduced influx peak at 200 nm from root tip (red line) that is shifted apically.
(C) Root and shoot free IAA concentrations of BUM- (0.5 µM) and NPA-treated (5 µM) wild type seedling. Data are mean ± SD (n = 4 with each 40-50 seedlings); absolute wild-type values were 42.2 ± 5.7 and 49.9 ± 6.1 pg/mg (fresh weight) for roots and shoots, respectively. Significant differences (unpaired *t* test with Welch's correction, $p < 0.05$) between inhibitor and solvent treatments are indicated by asterisks.

Fig. 4: Effect of BUM on PIN and ABCB abundance and locations
Localization of PIN-GFP, PIN2-GFP LIT, ABCB1-GFP and ABCB19-GFP MRAVEC fusion proteins (green)

in Arabidopsis roots 5 dag upon BUM (0.5 µM) and NPA (5 µM) treatments (24h). Note enhanced non-polar PIN1 signals in PIN2 locations upon BUM (inset) and NPA treatments, and reduced PIN2 expression upon NPA treatment in the elongation zone. Root borders are marked in red.

Fig. 5: BUM binds to and inhibits ABCB auxin exporters

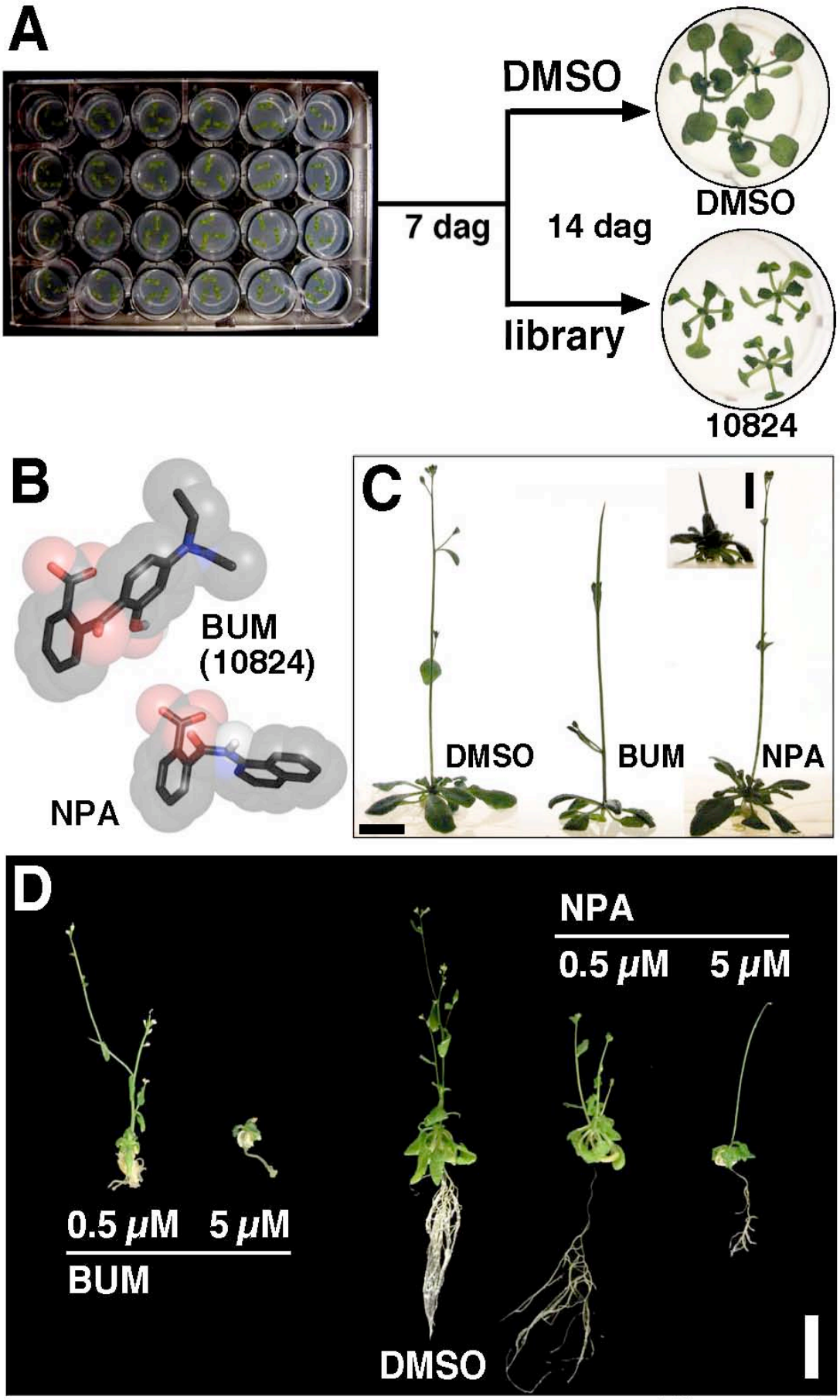
(A) BUM specifically inhibits ABCB1-mediated IAA but not benzoic acid (BA) export in yeast (mean ± SD; n = 4). PIN2-mediated export was 109.2 ± 19.5% of ABCB1 activity.

(B) BUM competes for NPA-binding to wild type and *pin2* microsomes but to lesser extent in *abcb1* membranes (mean ± SD; n = 4).

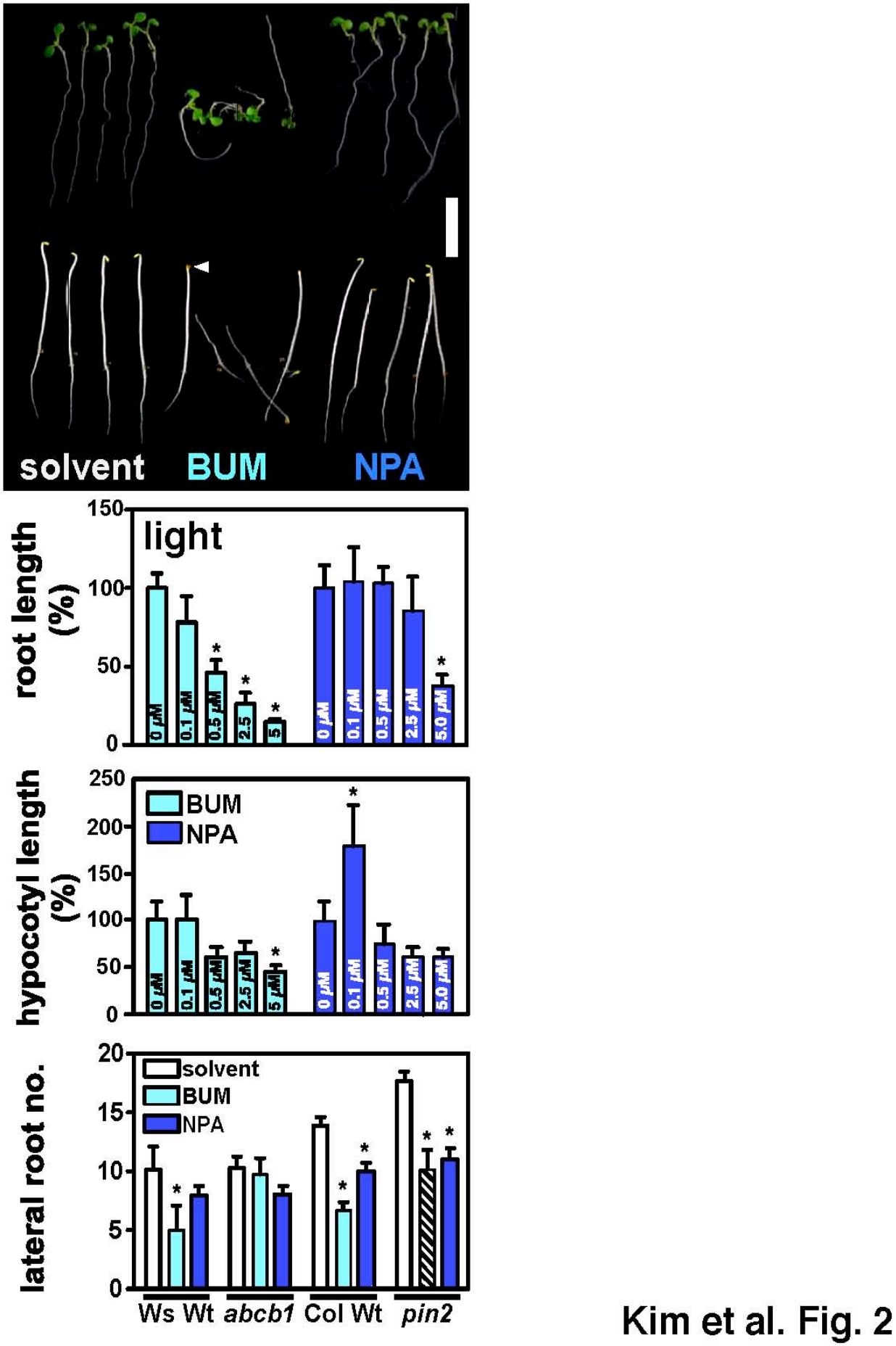
(C) *In silico* drug binding to the ABCB1 NBDs suggest overlapping and distinct inhibitor binding pockets for BUM (light blue) and NPA (dark blue). Note that NPA docks to pockets flanked by coupling helices (red) and Q loop (orange) of NBD1 and NBD2 while BUM docks only to the pocket corresponding to NBD2.

(D) BUM, like NPA (each 5 µM), disrupts TWD1-ABCB1 interaction monitored by yeast BRET assays while gravacin had only minor effects (mean ± SE; n = 4).

Significant differences (unpaired *t* test with Welch's correction, $p < 0.05$) between inhibitor and solvent treatments are indicated by asterisks.



Kim et al. Fig. 1



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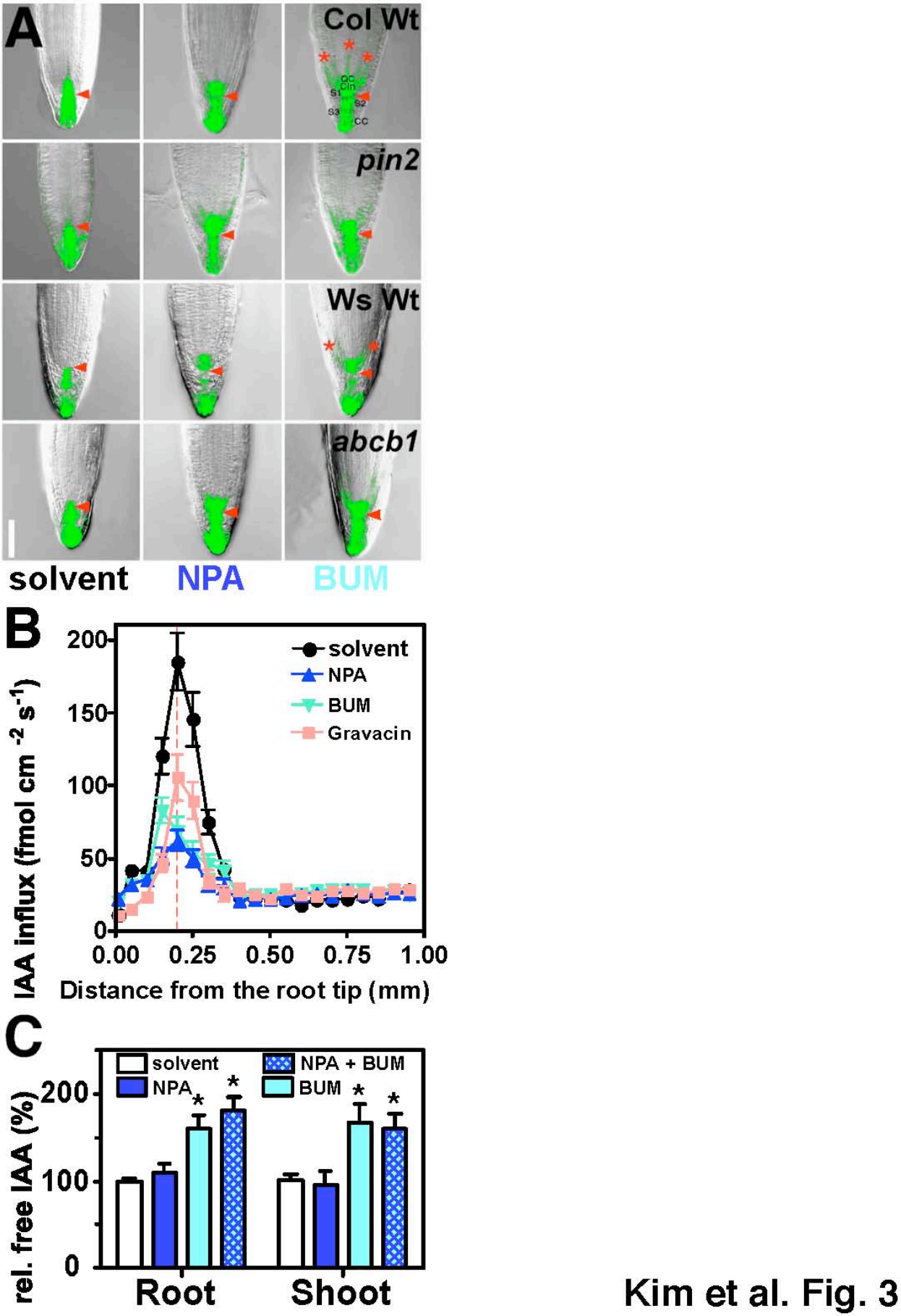

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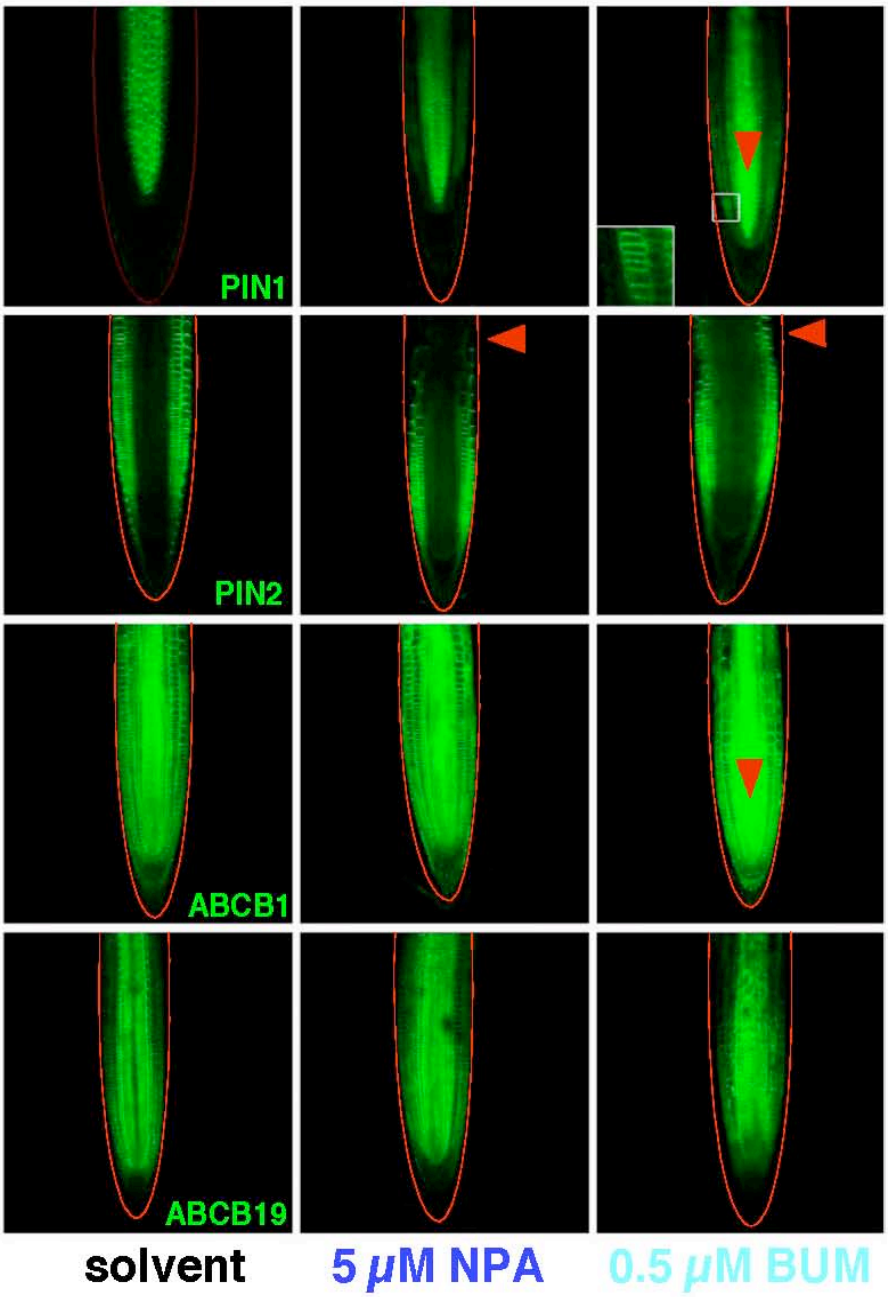

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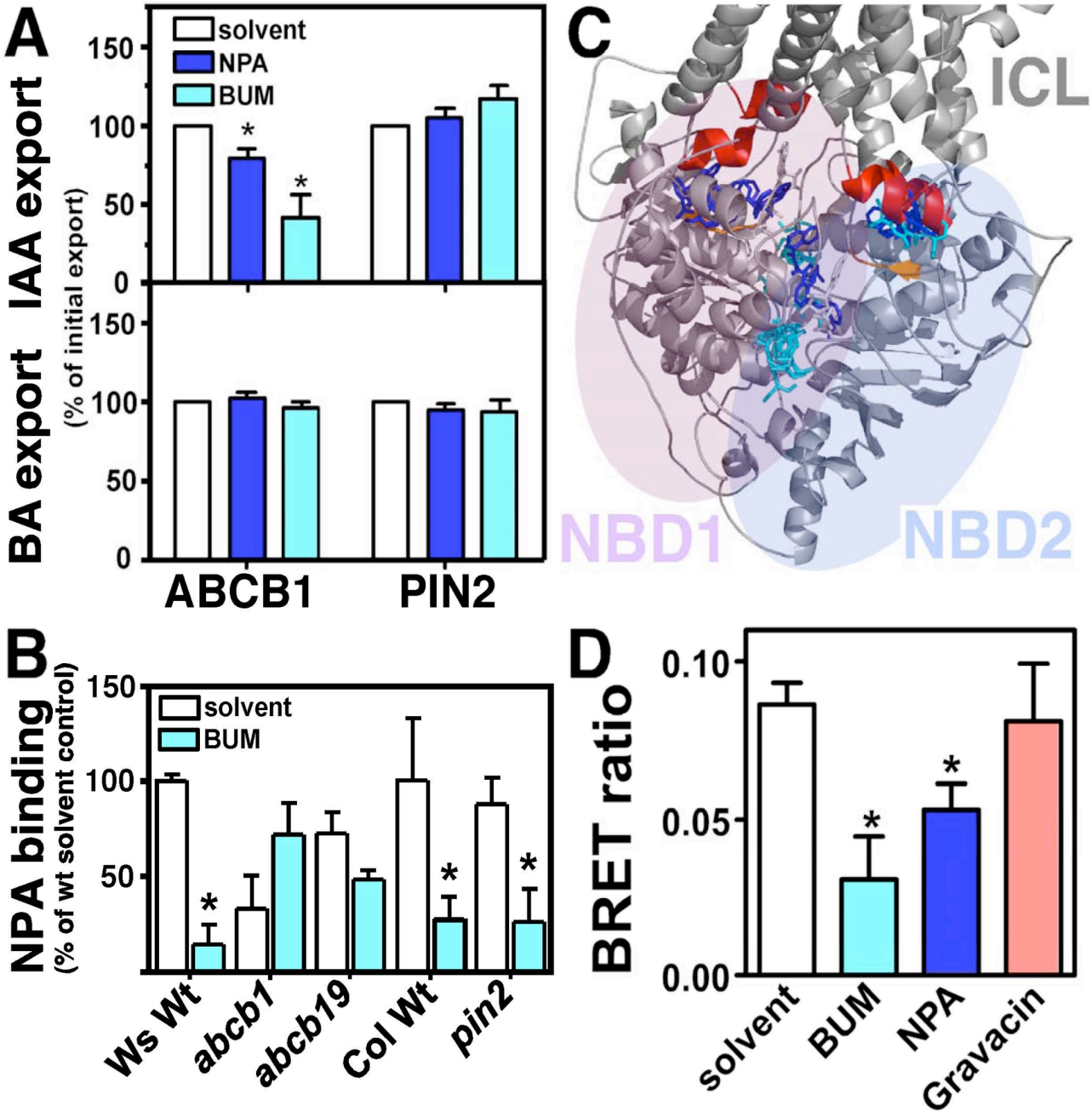

Kim et al. Fig. 5

Supplemental Data

Identification of an ABCB/P-glycoprotein-specific inhibitor of auxin transport by chemical genomics

Jun-Young Kim [1, *], Sina Henrichs [2, *], Valpuri Sovero [2], Aurélien Bailly [2], Stefano Mancuso [3], Stephan Pollmann [4], Daehwang Kim [5], Markus Geisler [2] and Hong-Gil Nam[1]

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Supplemental Materials and Methods

Quantification of root gravitropism

5 dag seedlings were transferred for an additional 12h onto plates containing 0.5 µM, 5 µM NPA or BUM or the solvent DMSO. Plates were rotated 90° from the vertical for 12h of gravity stimulation in the dark as in (39). The angle of root tips from the vertical plane (root curvature) was determined using Photoshop 7.0. (Adobe Systems). The number of seedlings for each genotype was between 72 and 96.

Isolation of Total RNA and RT-PCR Analysis

Seedlings were grown in light for 7 days and then incubated in the presence or absence (solvent control) of 0.1, 1 or 10 µM BUM or NPA for 3 hours. Total RNA was isolated from roots using the TRIzol reagent (WelGENE Inc., Deagu, Korea) according to the manufacturer's instructions. One µg of total RNA was used for first-strand cDNA synthesis with the ImProm-II Reverse Transcription system (Promega, Madison, WI) and then was subjected to PCR amplification (30 s at 95°C, 30 s at 55°C, 1 min at 72°C; 28 cycles) using the following sets of primers. ABCB1-UP: 5'- gaagaagttctcaagtatgcg; ABCB1-LP: 5'-agatctttgcagcagcaacc; ABCB19-UP: 5'-attgccgtgatacagcaagg; ABCB19-LP: 5'-ttcagagatagttgctgagc; PIN1-UP: 5'-ccttcgaatctaaccaacgc; PIN1-LP: 5'-agactgaacatagccatgcc; PIN2-UP: 5'-aactcctccatgataacgcc; PIN2-LP 5'cactcattatcgttggcatct; PIN3-UP: 5'-aacgggtcacccgtttcag; PIN3-LP: 5'-agtatcagccttgtcatcac; PIN4-UP: 5-gtcatccagctcattgcttg; PIN4-LP: 5'-ggcagggatgatatagacgg-3' AUX1-UP: 5'-attcagctctgcgatctccg; AUX1-LP: 5'-tggtgaccaatgtggcaaa; ACT8-UP: 5'-

aatcagatgtggatctctaaggca; ACT8-LP: 5'-tccgagtttgaagaggctacaaac. PCR products were fractionated in 1% (w/v) agarose gels and stained with ethidium bromide. The *ACTIN8* (*ACT8*) transcript level was employed for normalization.

Supplemental Figure Legends

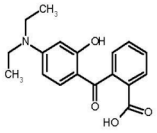
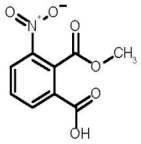
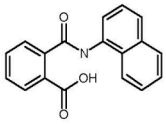
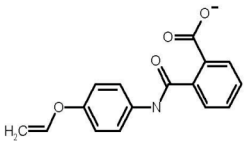
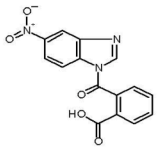
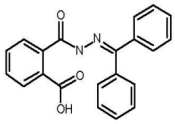
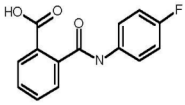
Fig. S1: Ability of BUM analogs to produce pin-shaped inflorescenses.
In a secondary screen, plants were treated with various concentrations (up to 10 µM) with selected compounds taken from the Korea Chemical Bank library that contained like BUM and NPA the 2-(formyl)-benzoic acid core (Fig. 1B). Phenotypes were screened for induction of pin-formed inflorescences (see Fig. 1C).

Fig. S2: BUM disrupts gravitropic root responses.
Root curvature of *abcb1/pgp1* and *pin2/eir1-4* alleles in comparison with corresponding wild type ecotypes (Ws Wt and Col Wt, respectively) in the presence (lower row) and absence of BUM each (upper row; 0.5 µM). Root curvatures were assigned to one of twelve 30° sectors in the circular histograms (see scheme). The length of each bar represents the percentage of seedlings showing the same direction of root growth; numbers in histograms are percent occurrence of 90 and 60° bending (sum of 60°, 90° and 120° sectors). Data are means (n= 3 with each 50–60 seedlings).

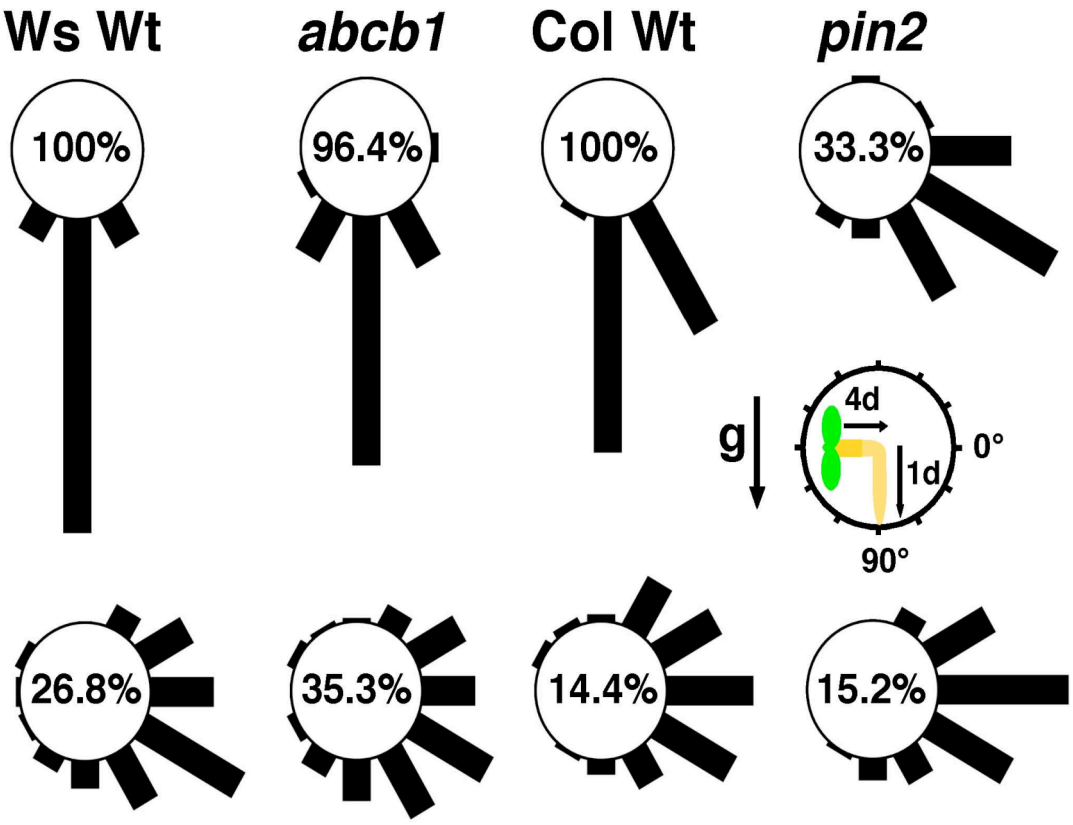
Fig. S3: Effect of BUM and NPA on expression of auxin transporter transcripts in root.
Wild type *Arabidopsis* seedlings were grown in half B5 media and transferred to new half B5 media including BUM and NPA, indicated root auxin transporter transcripts were quantified by semi-quantitative RT-PCR using *ACTIN8* as internal control.

Fig. S4: Reduction of primary root and hypocotyl lengths and lateral root numbers by BUM is less pronounced in the dark.
Data are mean ± SD (n = 3 with each 20-30 seedlings); significant differences (unpaired *t* test with Welch's correction, $p < 0.05$) between inhibitor and solvent treatments are indicated by asterisks. Absolute root and hypocotyl lengths were 8.3 ± 0.8 mm and 13.2 ± 1.0 mm, respectively.

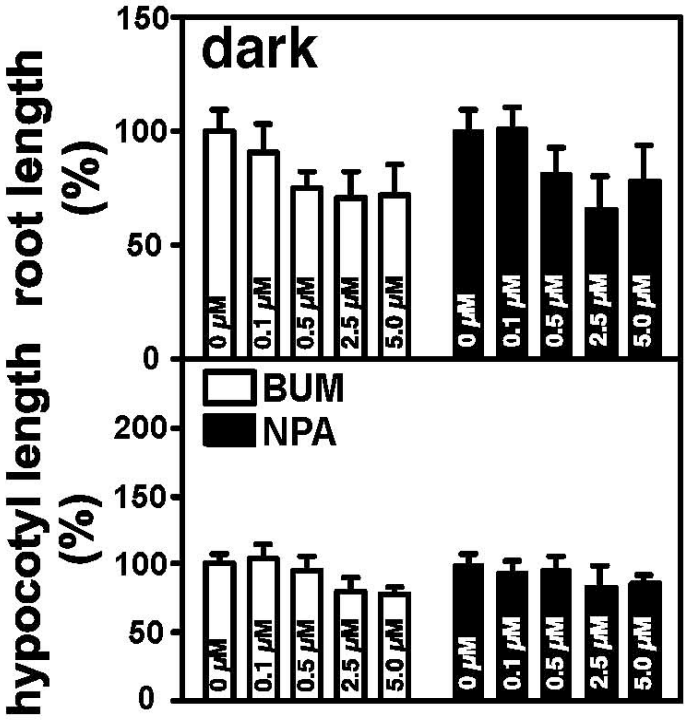
Fig S5. Dose-dependent responses of *Arabidopsis* primary root length to BUM and NPA
Wild type seedlings (Col) were cultured vertically on medium containing various concentration of BUM and NPA. The concentration ranges of BUM and NPA were and 0 - 20 µM and 0 - 28 µM, respectively. Primary root length was measured after 7 dag and IC_{50} values were calculated using sigmoidal dose-response fits. Data are mean ± SD (n = 3 with each 20-30 seedlings).

Name in study	Code in Korea Chemical Bank (K) or ChemBridge library (CB)	Chemical structure	Pin-formed inflorescences at 1 μM
BUM (10824)	K8617		Yes
A	KM00396		No
B	K12035		No
C	CBC0079		No
D	CBC0171		No
E	CBI0316		No
F	CBM0575		No

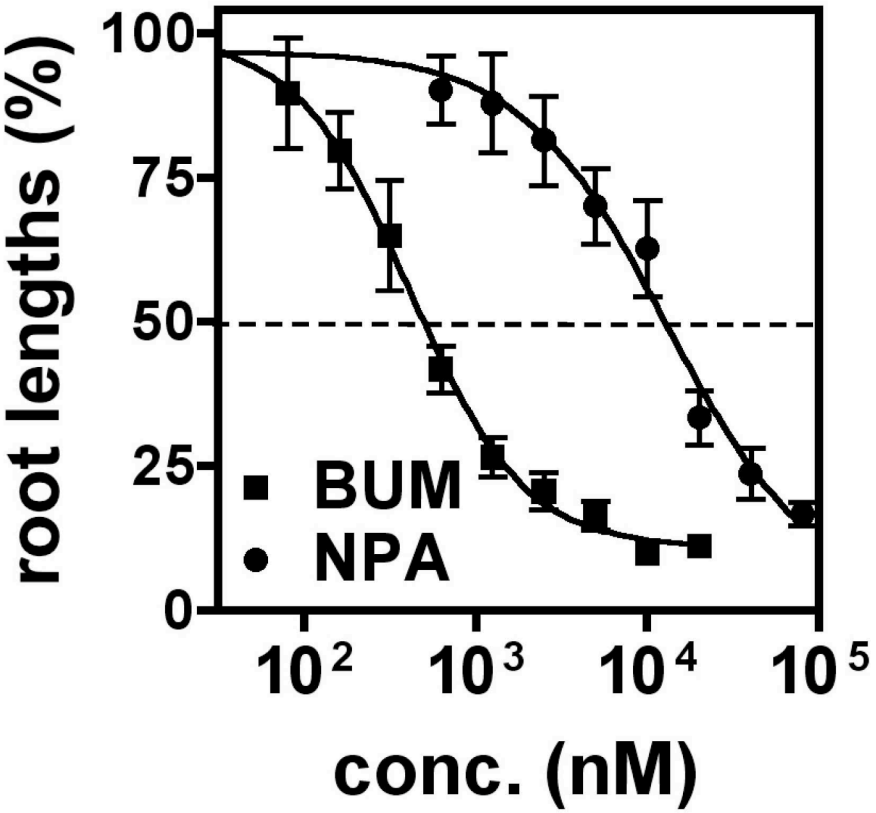
Kim et al. Fig. S1



Kim et al. Fig. S2



Kim et al. Fig. S4



Kim et al. Fig. S5

5.3 The AGC kinase PINOID mediates quercetin-dependent, counter-regulation of ABCB- and PIN-catalyzed auxin transport

Sina Henrichs, Yoichiro Fukao, Bangjun Wang, Matthias Weiwad, Stephan Pollmann, Stefano Mancuso, Masayoshi Maeshima, Alexander Schulz and Markus Geisler

Abstract

Polar auxin transport is controlled by the independent and coordinative action of PIN- (PIN-FORMED) and ABCB/PGP (P-GLYCOPROTEIN) efflux catalysts. PIN polarity was shown to be regulated by the AGC protein kinase, PINOID (PID), while ABCB activity is controlled by physical interaction with the immunophilin-like FKBP42, TWISTED DWARF1 (TWD1).
Using tandem-affinity purification (TAP) technology followed by shotgun proteomics, we identified PID as a valid TAP-TWD1 interactor of the TWD1/ABCB auxin efflux complex. *In vitro* pull-downs and BRET imaging *in planta* confirmed TWD1-PID interaction. Co-expression in yeast indicates that PID specifically modulates ABCB1 and ABCB1-PIN1,2 auxin efflux that is dependent on its kinase activity. A detailed analysis in tobacco revealed that PID inversely regulates ABCBs and PIN-mediated auxin efflux. *PID* loss-of-function mutants show enhanced cellular auxin efflux, respectively, resulting in altered root and shoot auxin levels that are inline with reported developmental defects. The known inhibitor of auxin transport and protein kinases, quercetin, but not NPA reverts specifically PID inhibition of ABCB1 activity by binding to and inhibiting PID auto-phosphorylation.
In summary, we provide evidence that PID phoshorylation does not only determine PIN polarity but has also a direct impact on transporter activity in an action that is reversed by modulators of auxin transport, like quercetin.

Own contribution

Figure 2: PID modulates ABCB- and PIN-mediated auxin efflux in yeast.
Yeast auxin export assays of different protein combinations for IAA export in *S.cerevisiae*.

Figure 3: The protein kinase and auxin transport inhibitor, quercetin, blocks PID-mediated ABCB1 inhibition.
Yeast auxin export assays of different protein combinations for IAA export in *S. Cerevisiae* in the presence of different kinase inhibitors.

Figure 4: Quercetin binding blocks PID kinase activity.
(A) *In vitro* auto-phosphorylation assay of PID-GST in the presence of quercetin, chelerythrine, IAA and NPA. Quantification of signal intensities.
(C) Binding assay for NPA and quercetin to plant microsomes.

Figure 5: PID counter-actively regulates ABCB1- and PIN1-mediated auxin efflux *in planta*
(A) Auxin efflux assay in Arabidopsis protoplasts.
(B) Sample preparation for root and shoot free IAA concentrations measurements by chromatography-mass spectrometry (GC-MS) of BUM- and NPA-treated wild type seedlings.
(C) Co-transfection of *N. benthamiana* leaves and auxin export assay from mesophyll protoplasts.
(D) Confocal microscopy analysis and imaging for the protein localization of 35S::PIN1:YFP and 35S::ABCB1:YFP in the presence of 35S::PID:GFP in tobacco protoplasts.

Figure 6: Protein kinase inhibitors, like quercetin and chelerythrin, block root PAT but rescue the *pin2* agravitropic phenotype.
(B) Gravitropic Assays of different mutant genotypes in the presence of kinase inhibitors.

Figure S2: PID modulation of ABCB- and PIN-mediated IAA export is specific.
(A+B) Yeast auxin export assays of different protein combinations for BA export in *S.cerevisiae*.
(C) Confocal microscopy analysis and imaging for the protein localization of 35S::ABCB1:YFP

in the presence of 35S::PID or 35S::MPID in yeast cells. Detailed analysis by the Imaris programme.

Figure S3: Quercetin as well as mammalian protein kinase inhibitors, chelerythrine and staurosporine, have no significant effect on ABCB1 mediated BA export both in the absence and presence of PID.
Yeast auxin export assays of different protein combinations for BA export in *S. Cerevisiae* in the presence of different kinase inhibitors.

Figure S4: Quercetin binding blocks trans-phosphorylation of MBP by PID
In vitro phosphorylation assay of MBP by PID-GST in the presence of quercetin, chelerythrine, IAA and NPA. Quantification of signal intensities.

Figure S5: Time-course of auxin efflux from *PID* gain- and loss of function protoplasts.
Co-transfection of *N. benthamiana* leaves and auxin export assay from mesophyll protoplasts.

Figure S6: Staurosporine and phorbole ester do not rescue the *pin2* agravitropic phenotype.
Gravitropic Assays of different mutant genotypes in the presence of kinase inhibitors.

Figure S7: Protein kinase inhibitors do not significantly alter ABCB1-, ABCB19, PIN1- and PIN2 root locations.
Confocal microscopy analysis and imaging for the protein localization of ABCB1::ABCB1:GFP, ABCB19::ABCB19:GFP, PIN1::PIN1:GFP, PIN2::PIN2:GFP in roots.

Title page

The AGC kinase PINOID mediates quercetin-dependent, counter-regulation of ABCB- and PIN-catalyzed auxin transport

Sina Henrichs[1], Yoichiro Fukao[2], Bangjun Wang[1,3], Matthias Weiwad[4], Stephan Pollmann[5], Stefano Mancuso[6], Masayoshi Maeshima[7], Alexander Schulz[3], Markus Geisler[1,8]

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Key words: polar auxin transport, ABCB, PIN, PINOID, quercetin, protein kinase

Abstract

Polar auxin transport is controlled by the independent and coordinative action of PIN- (PIN-FORMED) and ABCB/PGP (P-GLYCOPROTEIN) efflux catalysts. PIN polarity was shown to be regulated by the AGC protein kinase, PINOID (PID), while ABCB activity is controlled by physical interaction with the immunophilin-like FKBP42, TWISTED DWARF1 (TWD1).

Using tandem-affinity purification (TAP) technology followed by shotgun proteomics, we identified PID as a valid TAP-TWD1 interactor of the TWD1/ABCB auxin efflux complex. *In vitro* pull-downs and BRET imaging *in planta* confirmed TWD1-PID interaction. Co-expression in yeast indicates that PID specifically modulates ABCB1 and ABCB1-PIN1,2 auxin efflux that is dependent on its kinase activity. A detailed analysis in tobacco revealed that PID inversely regulates ABCBs and PIN-mediated auxin efflux. *pid* loss-of-function mutants show enhanced cellular auxin efflux, respectively, resulting in altered root and shoot auxin levels that are inline with reported developmental defects. The known inhibitor of auxin transport and protein kinases, quercetin, but not NPA reverts specifically PID inhibition of ABCB1 activity by binding to and inhibiting PID auto-phosphorylation.

In summary, we provide evidence that PID phoshorylation does not only determine PIN polarity but has also a direct impact on transporter activity in an action that is reversed by modulators of auxin transport, like quercetin.

Introduction

Plant development and physiology is widely controlled by a unique, plant specific process, the cell-to-cell or polar transport of auxin (PAT) that is controlled by members of the PIN- (PIN-FORMED) and B subfamily of ABC transporter, ABCB/PGP/MDR (P-GLYCOPROTEIN, MULTIDRUG-RESISTANCE).
PIN efflux carriers show polar locations in PAT tissues, developmental, organogenetic loss-of-function phenotypes and are thought to be the determinants of a “reflux loop” in the root apex (Blilou et al., 2005; Vieten et al., 2007). ABCB isoforms have been identified as primary active (ATP-dependent) auxin pumps showing auxin-related, developmental (but not organogenetic) loss-of-function phenotypes (Geisler et al., 2005; Blakeslee et al., 2007; Mravec et al., 2008a). Despite their mostly apolar locations, they have been demonstrated to contribute to PAT and long-range auxin transport (Geisler et al., 2005; Bouchard et al., 2006; Bailly et al., 2008). Moreover, ABCB1/PGP1 and ABCB19 coordinately function in basipetal reflux of auxin maxima out of the root and shoot tip (Blakeslee et al., 2007). The immunophilin-like FKBP42, TWISTED DWARF1 (TWD1), was characterized as a central regulator of ABCB-mediated auxin transport by means of protein-protein interaction (Geisler et al., 2003; Abas et al., 2006; Geisler and Bailly, 2007). Positive regulation of ABCB1/PGP1- and ABCB19/PGP19/MDR1-mediated auxin transport accounts for overlapping phenotypes between *twd1* and *abcb1 abcb19* (Bouchard et al., 2006; Bailly et al., 2008).

Independently, ABCB1 and ABCB19 have been identified as targets of the synthetic auxin transport inhibitor (ATI), 1-*N*-Naphtylphtalamic acid (NPA) (Geisler et al., 2005; Rojas-Pierce et al., 2007; Nagashima et al., 2008). Several lines of evidence suggest that PIN proteins do not itself act as NPA Binding Proteins (Lomax et al., 1995), but additionally NPA binds to TWD1 and NPA binding disrupted TWD1-ABCB1 interaction (Bailly, 2008a). *In planta* this leads to disruption of ABCB1 activity, suggesting that TWD1 and ABCB1 represent high- and low-affinity components of the NPA-sensitive efflux complex (Bailly, 2008a).
ABCB- and PIN-mediated auxin efflux can function independently and play identical cellular but separate developmental roles (Mravec et al., 2008a). However, ABCBs and PINs are also able to interactively and coordinately transport auxin (Blakeslee et al., 2007). The current picture that emerges is that in interacting cells, multilaterally expressed ABCBs minimize apoplastic reflux while polar ABCB-PIN interactions provide specific vectorial auxin stream (Mravec et al., 2008a).

Reversible protein phosphorylation is a key regulatory mechanism for PAT (Delbarre et al., 1998) and the best-investigated example is the phosphorylation-dependent polar targeting of PIN proteins. This process is antagonistically regulated by the serine-threonine kinase PINOID (PID) and the trimeric serine-threonine protein phosphatase 2A (PP2A). Beautiful correlation between *in vivo* phosphorylation studies and PIN locations suggests that PID and PP2A antagonistically determine the fate of PIN cargoes for trafficking to the appropriate membrane by phosphorylating the hydrophilic loop of PIN proteins (Michniewicz et al., 2007).
The regulatory A subunit, PP2AA1, called *ROOTS CURL IN NPA1* (*RCN1*), causes various developmental defects, including root agravitropism, cotyledon defects and

root meristem collapse (Garbers et al., 1996; Deruere et al., 1999). *Rcn1* was isolated in a screen for alterations in differential root elongation in the presence of NPA. PP2A is a negative regulator of basipetal transport in the root and as a consequence *rcn1* roots exhibit a significant delay in gravitropism, consistent with an increased basipetal auxin transport (Rashotte et al., 2001; Sukumar et al., 2009). Importantly, the *rcn1* gravitropic phenotype can be rescued by low concentrations of NPA, a concentration that is sufficient to block gravitropism in wild-type seedlings (Muday and DeLong, 2001). On the other hand, acropetal auxin transport is unaffected in *rcn1*, but shows a dramatic loss of NPA inhibition. Interestingly, *rcn1 pin2* double-mutant analyses indicate that elevated basipetal transport in *rcn1* does not require PIN2, leading to the suggestion that an NPA-binding protein is involved in this process (Rashotte, DeLong et al. 2001).

PID belongs to the AGC family of serine/threonine kinases, named after their functional mammalian othologues, PKA, PKG and PKC. Among the subfamily AGCVIII, PID groups to the AGC3 clade together with AGC3-4, WAG1 and WAG2 (Galvan-Ampudia and Offringa, 2007). Loss-of-function mutations of *wag1 wag2* show an auxin-dependent root waving phenotype and root curling is more resistant to NPA (Santner and Watson, 2006). This together with the fact that WAG kinases (like PID) are plasma membrane-associated suggested that PID and WAG kinases act in the same or in a parallel regulatory pathway of PAT (Galvan-Ampudia and Offringa, 2007).

PID loss- or *35S:PID* gain-of-function changes the apical (shoot-tip-facing) or basal (root-tip-facing) cellular localization of PIN proteins influencing the direction of the auxin movement (Friml et al., 2004). As a consequence, in the *pid* mutant PIN1 localizes to the basal membrane of epidermal cells, which in turn redirects auxin away from the meristem and prevents the initiation of new lateral organs, thus resulting in the pin-shaped inflorescence (Christensen et al., 2000). In the root tip, the *pid* mutation causes endomembrane accumulation of PIN2 without a shift in PIN2 polarity (Sukumar et al., 2009). On the other hand PID over-expression leads to basal-to-apical switch of PIN1, PIN2 and PIN4 in root cortex and lateral root cap cells and finally to a collapse of the root meristem due to auxin depletion (Friml et al., 2004).
Mutant analysis and pharmacological treatment with kinase/phosphatase inhibitors, staurosporine/cantharidine, revealed that PID and PP2A antagonistically regulate basipetal auxin transport and gravitropic response in the root tip (Sukumar and Edwards et al. 2009). Loss of PID activity alters the PIN2-mediated basipetal auxin transport and impedes the gravitropic response, without causing an obvious change in PIN2 cellular polarity. This finding indicates that PID promotes and enhances root gravitropism, but is not absolutely required. Furthermore, PID appears to have a specific regulatory effect on the basipetal transport machinery in the root, since the acropetal transport is unaffected in *pid*. This goes in hand with a polar localization of PID-YFP in the epidermal and cortical cell layers that partially overlap with PIN2 (Michniewicz et al., 2007).

All these data are consistent with the concept of PID as a positive regulator of IAA efflux (Benjamins et al., 2001; Lee and Cho, 2006). However, the direct affect of PID

on individual transporter activities has not yet been addressed. Here, we identify and characterize PID as TWD1 interactor. Our data suggest that PID, beside its function as a molecular switch of PIN polarity, has a direct impact on PIN- and ABCB-type auxin efflux transporter activity. PINs and ABCBs are counter-regulated by PID in an action that is reversed by binding of protein kinase inhibitor, quercetin, a modulator of auxin transport.

Results

Identification of PINOID as an interactor of the ABCB-TWD1 auxin efflux complex

With the aim of identifying novel components of the auxin efflux complex, characterized by ABCB1 and TWD1, we employed the tandem-affinity purification (TAP) method followed by shotgun mass spectrometry analysis of TAP-tagged TWD1 (TAP-TWD1). We chose the TAP procedure that has been successfully used for the completion of the *S. cerevisiae* interactome (Pu et al., 2007) and also for the identification of several plant protein interactions (Abe et al., 2008; Van Leene et al., 2008), because of its high degree of two-step affinity purification and the use of established purification protocols. As starting material, we used total microsomes of roots of Arabidopsis seedlings for the two-step TAP purification reducing typical non-specific contaminants (like ribosomal proteins, chaperons and Rubisco) (Table S1). To further gain specificity of interacting proteins, we subtracted identified vector control proteins from TAP-TWD1 proteins, and proteins with a score above 30 were considered as significant interactors (Fig. 1A). This procedure was repeated with each two independent transformants resulting in essentially the same TWD1 interactors.

Beside TWD1 as an obvious dominant pulled-down protein (protein score of 129, 13.4% coverage; Fig. 1A), we found an uncharacterized polynucleotide adenylyltransferase-like protein (At3G48830), the unknown protein At4G25920, the histidin-like kinase AHK5/CYTOKININ INDEPENDENT2 (At5G10720) that regulates root elongation (Iwama et al., 2007), the ACG kinase PINOID (At2G34650) and the catalytic domain of the PP2C-type protein phosphatase, AP2C1 (At2g30020). While AP2C1 is known to be involved in innate immunity responses by the negative regulation of the MAP kinases MPK4 and MPK6 and further jasmonic acid, and ethylene levels in Arabidopsis (Schweighofer et al., 2007), PINOID (PID) is a well known key player in polar auxin transport regulation (Benjamins et al., 2001; Friml et al., 2004; Lee and Cho, 2006; Mravec et al., 2008a) and was therefore chosen for further analysis.

Identification of PID as TAP-TWD1 interactor does not necessarily imply direct physical interaction as the PID-TWD1 interaction might also be mediated via a third TWD1 interacting protein. In order to test this possibility and to verify the TAP data, we performed *in vitro* pull-down experiments using recombinant PID-GST and $TWD1^{1-337}$ protein. Indeed, PID-GST but not GST alone or the mock control was able to pull-down small but significant amounts of $TWD1^{1-337}$ (Fig. 1C), which is resulting typically in a double band (Geisler et al., 2003). TWD1-PID interaction was further substantiated by *in planta* Bioluminescence resonance energy transfer (BRET) measurements (Bailly et al., 2008) by expressing rLuciferase- and YFP-tagged versions of TWD1 (TWD1-rLuc) and PID (PID-YFP), respectively, in *N. benthamiana* leaves (Fig. 1B). Co-transfection of TWD1-rLuc and PID-YFP, but not single combinations or YFP alone, resulted in low but significant BRET ratios, and indicative for physical proximity below 100Å and thus interaction.

In summary, our results demonstrate the utility of the TAP method for discovering valid protein–protein interactions of membrane complexes in Arabidopsis and suggest a relevant TWD1-PID interaction *in planta*.

PID counter-regulates ABCB1- and PIN1-mediated auxin efflux

PID is currently seen as a molecular switch that defines polar PIN locations and thus the directionality of auxin streams most probably by direct PIN phosphorylation that is thought to be reversed by PP2A phosphatases (Michniewicz et al., 2007). A comparable mechanism has so far not been demonstrated for ABCBs, whose cellular localization is mainly non-polar, or for TWD1, who functions during the regulation of ABCB1 efflux activity (Bouchard et al., 2006; Bailly et al., 2008). Therefore, we quantified ABCB1-mediated auxin efflux in the presence and absence of MYC-tagged PID in yeast. Surprisingly, PID reduced significantly ABCB1 but not the vector control (background) IAA efflux by ca. 30% (Fig. 2A), similarly to what is found for TWD1 co-expression. This inhibitory effect caused by PID was specific as it was not found with the mutated, kinase-negative MPID (Christensen et al., 2000) or the non-related GSK-3-like kinase BRASSINOSTEROID-INSENSITIVE2 (BIN2), a negative regulator of brassinosteroid (BR) signaling and cell elongation (Li et al., 2001; Li and Nam, 2002; Vert et al., 2008). Moreover, PID or MPID co-expression did not substantially alter ABCB1 expression or localization in yeast (Fig. S2C).

Next, we tested if PID had an effect on the PIN1 or PIN2 mediated auxin transport alone and on ABCB1-PIN1,2 combinations, that are known to functionally interact in yeast (Blakeslee et al., 2007). While PID did not significantly influence the PIN1 or PIN2 mediated transport alone, it blocked the ABCB1-PIN1- and ABCB1-PIN2 efflux drastically back to vector control (background) level. This is again not seen with the inactive form of PID (MPID) (Fig. 2B). Remarkably, all PID induced inhibitions were specific for IAA transport, as they were not found for the unspecific transport control, benzoic acid (BA) (Fig. S2).

To further test our yeast-based results *in planta*, we measured auxin efflux in *N. benthamiana* mesophyll protoplasts that were co-transfected with PID-ABCB1 or -PIN1. In contrast to the yeast system, where PIN1 alone (without ABCB) was shown to be inactive (Fig. 2B; (Blakeslee et al., 2007), PIN1 expression in tobacco cells resulted in a high auxin efflux, which was roughly twice of what was found for ABCB1 (Fig. 5C). Interestingly, co-expression with PID resulted in a drastically inhibited PIN1-catalyzed export nearly back to vector control level. Interestingly, and in contrast to what was found in yeast, PID activated the ABCB1 auxin efflux by a factor 2-3, suggesting an inverse mode of regulation on ABCB1- and PIN1-catalyzed auxin efflux *in planta*. In addition, PID expression alone enhanced vector control IAA and NAA efflux slightly, most probably by activating the tobacco endogenous ABCB-type exporters. In analogy to the yeast system, PID expression did not alter ABCB1 or PIN1 expression or location on the plasma membrane as monitored by co-localization analysis of GFP-tagged PID and YFP-tagged ABCB1 and PIN1 (Fig. 5D). These results provide evidence that PID counter-actively regulates PIN- and ABCB auxin efflux in a mode that requires its kinase activity.

The protein kinase inhibitors, quercetin and staurosporin, revert PID-mediated ABCB1 modulation

A recent publication suggests PID as a primary target for staurosporine, a well-known kinase inhibitor, and application of low concentrations to wild-type seedlings phenocopies the reduced basipetal auxin transport and delayed gravitropic response of *pid* (Sukumar et al., 2009). To further substantiate the mechanism underlying PID-mediated ABCB1 regulation, we tested therefore the effect of different protein kinase inhibitors, like chelerythrine, staurosporine and quercetin on PID regulation. Chelerythrine is a potent and selective inhibitor of PKCs (IC_{50} = 0.7uM), while staurosporine is known to be less specific but more potent (IC_{50} (PKA) = 60nM; IC_{50} (PKC) = 30nM). However, the flavonoid quercetin is thought to act as a modulator of PAT and was shown to inhibit ABCB auxin transport (Geisler et al., 2005; Terasaka et al., 2005; Bouchard et al., 2006) and to disrupt ABCB1-TWD1 interaction (Bailly et al., 2008).
Quercetin and staurosporine have only mild effect on vector control (background) or ABCB1 auxin efflux alone, while chelerythrine heavily enhances background activities independent of PID action (Fig 3). However, only quercetin and - to a less significant extend – staurosporine was able to efficiently revert PID-mediated ABCB1 inactivation, while the mammalian protein kinase inhibitor chelerythrine had only a mild effect. Again, reversal of drug inhibition was specific for IAA transport as the unspecific transport control BA assayed in parallel was not affected (Fig. S3).

Quercetin binds to and inhibits PID kinase activity
Yeast transport experiments suggested that regulation of ABCB and PIN transport activity by PID is coupled to its function as a kinase. As a consequence a functional PID protein in turn would be modulated by kinase inhibitors. To assess this concept we quantified PID auto-phosporylation and trans-phosphorylation of the standard kinase substrate, Myelin Basic Protein (MBP), in the presence and absence of protein kinase inhibitors and regulators of auxin transport. Quercetin blocked PID auto-phosphorylation (Fig. 4A) as well as MBP trans-phosphorylation (Fig. S4A) in a concentration dependent manner (0-1 μM) visualized in comparison to the according Coomassie stains (left panels) and autoradiographies of the phosphorylated MBP (Fig. S4) and PID bands (PID-P; Fig. 4). In contrast to the former yeast transport experiments, a similar magnitude of quercetin inhibition was also found for chelerythrine (each 1 μM), while IAA and the synthetic auxin transport inhibitor, NPA, showed no significant effects.
Chelerythrine and staurosporine have been shown to block PKC action by interacting with its catalytic domain (Herbert et al., 1990). In order to investigate if a similar mechanism is working during PID inhibition, we measured the binding of radiolabeled quercetin to purified PID-GST and GST alone. Analysis of specific binding (PID-GST minus GST) yielded in a significant quercetin binding while bound NPA, NAA and BA was neglectable (Fig. 4B). The small but significant IAA binding is currently under further investigation. The direct binding of quercetin to PID was further supported by the fact that microsomes from *PID* loss-of-function alleles showed drastically reduced quercetin binding (16.1 ± 33.1% of wild-type), while gain-of-function alleles (35S:PID) show a significantly higher signal (137.9 ± 45.5% of wild-type). Surprisingly, variation of *PID* expression had an inverse effect of NPA binding implying that PID, although itself apparently does not bind NPA, does alter NPA binding capacities of NPA-binding proteins.

In summary these data support the concept that PID is an *in vivo* target of quercetin that negatively regulates PID activity by direct drug binding.

Quercetin and chelerythrine block root PAT but rescue the *pin2* agravitropic phenotype.

To test the physiological relevance of kinase inhibitors affecting the PID kinase activity, as elaborated before in yeast and plant expression systems, we measured polar auxin transport in the root tip in the presence of quercetin and chelerythrine by using an auxin-specific electrode (Mancuso et al., 2005). The underlying method for measurements of root IAA influxes has, beside the indirect visualization of the auxin responsive element DR5, became a well-established and reliable tool to quantify auxin fluxes in the root (Santelia et al., 2005; Bouchard et al., 2006; Bailly et al., 2008). 5μM chelerythrine and quercetin resulted in a strong inhibition of the maximal influx peaks at 200μm from the root tip (Fig. 6A). This was also found for NPA at equal concentrations (Bailly et al., 2008) or genetically by loss-of *ABCB1*/*ABCB19* function alleles (Bouchard et al., 2006). Moreover, these data are inline with a recent report for staurosporine to reduce basipetal IAA transport and gravity response (Sukumar et al., 2009). Interestingly, quercetin and chelerythrine - unlike NPA treatments - lead to basal and apical shifts, respectively. Moreover, IAA influx in distal regions (between 0.3 and 1 mm from the root tip) is insensitive to chelerythrine treatment but sensitive to NPA and quercetin. This suggests overlapping targets responsible for maximum influx peaks but distinct target spectra for distal root regions as reported for *abcb1,19* and *twd1* loss-of-function roots (Bouchard et al., 2006; Bailly et al., 2008).

Recently, quercetin (like kaempferol) was shown to partially complement gravitropic-bending defects of *pin2* (*eir1-4*) roots in a PIN1-dependent fashion and thus not found for *pin1 pin2* alleles (Fig. 6B; Santelia et al. 2008). Not surprisingly, this rescue was also found for chelerythrine treatments (43.4% compared to 28.6%) at concentrations that do not inhibit root bending in wild-type seedlings, supporting the idea of a function for quercetin as an endogenous kinase inhibitor. In agreement, phorbole ester (21.4%), a potent PKC activator, shows a slight inhibitory effect on *pin2* (Fig. S6), leading to the suggestion that protein phosphorylation events trigger gravitropic root bending. Suprisingly, staurosporine (26.4% compared to 28.5%) was less effective during the *pin2* rescue, indicating alternative targets or mode of actions for the different kinase inhibitors. Unlike quercetin (Santelia et al., 2008) and chelerythrine which upregulate PIN1 expression at PIN2 domains (Fig. S7), staurosporine showed no influence on PIN1 expression or polar localization (Fig. S7; Sukumar et al., 2009). The fact that the rescue of *pin2* agravitropism was slightly enhanced in the presence of a combined quercetin and chelerythrine treatment (48.8% compared to 44.8% and 43.8%), argues for additive actions and independent pathways (Fig. S7). Further analysis showed that the chelerythrine rescue was <u>not</u> PIN1 dependent (Fig. 6B), which is in contrast to flavonols.

Remarkably, both quercetin and chelerythrine rescues were dependent on ABCB1 and ABCB19 functions, which became obvious by *pin2 abcb1 abcb19* triple mutant analysis (Fig. 6B), suggesting ABCB1 and ABCB19 as direct chelerythrine/ phosporylation targets.

In summary, these finding suggest that phosphorylation events trigger gravitropic root responses, that dependent on ABCB1 and ABCB19.

Discussion

Identification of PID as regulatory component of the auxin efflux complex

In this study, we have identified the AGC kinase PID by means of TAP-tagging as physical and functional interactor of the auxin efflux complex characterized by immunophilin-like TWD1, a regulator of ABCB-mediated auxin efflux. A typical limitation of this approach is that fusion proteins may not compete well with the endogenous protein, presumably due to steric hindrance from the TAP-tag domain. Therefore, and in analogy to our previous yeast BRET system where an N-terminally fusion was proven functional, we constructed an analog TAP-tagged TWD1 as bait. Therefore, we verified functionality of TAP-tagged TWD1 by genetic complementation test of the *twd1-3* allele with TAP-tagged TWD1. In all 8 tested lines, TWD1-TAP complemented the "twisted syndrome" in nearly all respects, except that 2 complemented alleles had a slightly reduced growth compared to wild-type (not shown). However, the scale of the planned experiments precluded testing both N- and C-terminally tagged versions. Biochemical analysis revealed that auxin-efflux capacities were restored to wild-type level by TAP-TWD1 complementation (not shown). TWD1-PID interaction was verified by BRET analysis *in planta* and *in vitro* pull-downs. Assuming a 1:1 PID-TWD1 ratio, pulled-down TWD1 was relatively low, suggesting that additive stabilizing or bridging factors were absent in the *in vitro* assay. Relevant candidates might be TOUCH3 (TCH3) or PINOID-BINDING PROTEIN1 (PBP1), which bind PID in a calcium-dependent manner, thus positively or negatively regulating its kinase activity, respectively (Benjamins et al., 2003). Of special interest is TCH3, a calmodulin-related protein (Benjamins et al., 2003), as calmodulin-binding to the TWD1 CaM-binding domain has been shown previously (Geisler et al., 2003; Kamphausen et al., 2002). Although the physiological relevance of this interaction is still unclear, studies on the human homologue of TWD1, FKBP38, show that function of HsFKBP38 requires a prior activation by calmodulin (Edlich et al., 2007).

Interaction with TWD1 of the efflux complex provides for the first time a plausible ratio for the soluble kinase that has no obvious (plasma) membrane association motifs (Galvan-Ampudia and Offringa, 2007) being a plasma membrane protein (Fig. 5; Friml et al., 2004; Michniewicz et al., 2007). Although, we cannot exclude an indirect mode of interaction provided by additional interactors, based on our data TWD1 provides a surface for PID recruitment resulting in phosporylation and thus regulation of catalytic components that control auxin efflux.

PID counter-regulates ABCB- and PIN transport probably by protein phosphorylation

A plasma membrane location for PID as shown here (Fig. 5), possibly by binding to phospholipids (Zegzouti et al., 2006), as well as interaction with TWD1 suggested a direct regulatory impact of PID on efflux activities. This was demonstrated by functional co-expression in yeast where PID was specifically, negatively blocking auxin efflux of ABCB1 as well as of ABCB1-PIN1 and ABCB-PIN2 complexes (Fig. 2). Results could be verified *in planta* using *N. benthamina* protoplasts as a novel expression system where PID expression, and more drastically, co-expression of

PID-ABCB1 resulted in enhanced auxin efflux (Fig. 5). These findings are in agreement with recent data describing enhanced efflux from BY-2 cells upon PID over-expression (Lee and Cho, 2006). They are, however, also inline with a very recent report that PID acts via PIN2 but that PIN2 polarity is unlike in 35S:PID widely unchanged in *pid.* This suggests that other targets are responsible for reduced basipetal transport in agravitropic roots (Sukumar et al., 2009). Interestingly, in contrast to ABCB1, PIN1 export was drastically blocked by PID co-expression *in planta* while ABCB1 and PIN1 locations and expression levels were unchanged. This indicates an opposite regulatory impact of PID on PIN- and ABCB auxin efflux catalysts. Discrepancies between PID effects on ABCB1 in yeast (inhibition) and plant systems (activation) have been found in analogy also for TWD1 modulation of ABCB1 (Bouchard et al., 2006; Bailly et al., 2008) and might be due to the lack of other regulatory components (exp. like TWD1), altered membrane composition in the lower eukaryote or simply caused by competition of PID with yeast-endogenous AGC kinases (Bailly et al., 2008; Yang and Murphy, 2009).
Regulation of PINs and ABCBs was specific as it was found for effluxed auxin but not BA controls and not mediated by unrelated GSK-3-like kinase, BIN2. Moreover, PID regulation of ABCB1 was dependent on PID kinase activity as kinase-deficient MPID had no effect and PID action was reverted by kinase inhibitors quercetin and staurosporine (Fig. 3). This is in agreement with data demonstrating PID to be staurosporine-insensitive providing evidence that PID is a direct target of staurosporine (Sukumar et al., 2009). Interestingly, both quercetin and staurosporine were shown to block PAT (Fig. 6; (Peer and Murphy, 2007) and gravitropism (Sukumar et al., 2009) and ABCB activity (Conseil et al., 2001; Geisler et al., 2005; Limtrakul et al., 2005; Terasaka et al., 2005; Blakeslee et al., 2007).
PID-mediated phosphorylation of PIN1 was shown *in planta* and additionally for PIN2 cytoplasmic loops *in vitro* (Michniewicz et al., 2007). *In vivo* proof for PID ABCB phosphorylation awaits confirmation but is likely as plant ABCB have been demonstrated in a phospho-proteomics approach to be phosphorylated in the regulatory linker domain (Nuhse et al., 2004). This stretch of roughly 60 amino acids is known to be phosphorylated by PKC and PKA analogs determining mammalian ABCB activity (Idriss et al., 2000).

The auxin transport inhibitor, quercetin, blocks PID activity by drug binding
Inhibitor treatment of phosphorylation and transport assays suggested that protein kinase inhibitors, chelerythrine, staurosporine and quercetin, block PID by inhibiting its kinase activity. Chelerythrine-quercetin and staurosporine-chelerythrine, have widely overlapping but partially also distinct dose-response and target spectra. These data are inline with a recent report suggesting PID as a primary target of staurosporine (Sukumar et al., 2009). Here, application of a low concentration of staurosporine to wild-type seedlings phenocopied the reduced basipetal auxin transport and delayed gravitropic response of *pid,* whereas *pid* seedlings are insensitive to the effect of staurosporine on root gravitropism. Staurosporine had no significant effect on PIN1,2 expression/locations (Fig. S7), indicating a direct effect on PIN activity. Discrepancies in the effect of staurosporine and chelerythrine on PID (as shown for yeast transport assays) might be at least partially caused by different specificities and the fact that, like staurosporine, also chelerythrine seemed to inhibit

transport activity of ABCBs dependent and independent of phosphorylation events mediated by PKC by binding to the transporter itself (Castro et al., 1999; Conseil et al., 2001).

Of special interest was quercetin, a well known clinical kinase inhibitor (Graziani et al., 1982; Graziani et al., 1983; Gschwendt et al., 1983) and modulator of auxin transport (Peer and Murphy, 2007). Quercetin, blocking efficiently PID action in a concentration-dependent manner, was specifically shown to bind to recombinant PID and *in planta.* This suggests a novel concept of auxin transport regulation where quercetin would block PID activity and thereby phosphorylation-dependent (in)activation of individual transporters by direct drug binding. Interestingly, disruption and thus inactivation by quercetin was recently also described for TWD1-dependent ABCB1 activation by means of protein-protein interaction (Bailly et al., 2008).

PID is not a direct target of NPA

Currently, PID is seen as a positive regulator of NPA-sensitive PAT. This is supported by the following findings: the pinoid mutant shoot phenotype can - in analogy to the more drastic one of *pin1* (Palme and Galweiler, 1999) - be widely phenocopied by NPA treatment (Wisniewska et al., 2006), *pid* shoots (Okamoto et al., 1991; Bennett et al., 1995) and roots (Sukumar et al., 2009) show reductions of acropetal and basipetal PAT, respectively, root defects of *35S:PID* allels can be rescued by NPA treatment (Christensen et al., 2000; Benjamins et al., 2001) and *pin1 pid* alleles show additive, drastic developmental defects (Furutani et al., 2007).
Here, we show that NPA has only a slight effect on PID kinase activity and does not bind to PID (Fig. 4 and 6). Surprisingly, although PID itself does not bind NPA, its loss- or gain-of-function does apparently alter inversely NPA binding capacities of NPA-binding proteins, like TWD1 or ABCBs (Bailly et al., 2008). These findings suggest that the *pinoid* phenotype and repression of 35S:PID defects by NPA are probably indirect. One plausible option is that PID phosphorylation of the ABCB-TWD1 complex might enhance NPA binding to ABCB1 and/or TWD1 (itself obviously not being phosphorylated by PID) resulting in ABCB inactivation.

Is PID a negative or positive regulator of auxin transport?

As explained above, several lines of evidence – including PID and PID/ABCB1 over-expression in tobacco protoplasts (Fig. 5C) - support a positive regulatory role of PID on auxin efflux. However, quantification of auxin efflux from *pid* leaf mesophyll protoplasts resulted in greatly enhanced efflux of native and synthetic auxins by roughly a factor 2 while *PID* over-expression had only slight effects (Fig. 5A). As a result, free IAA was elevated in entire 35S:PID roots and *pid* shoots (Fig. 5B). These data are in agreement with a DR5-GUS analysis of 35S:PID roots that revealed reduced IAA levels in the tips due to PIN switches but enhanced signals in distal parts with emerging lateral roots (Friml et al., 2004). They match obviously also the findings that *pid* roots (unlike *pid* shoots) show only a mild phenotype while the opposite holds true for 35S:PID alleles (Michniewicz et al., 2007). However, in summary, these data - generated in a shoot-derived single-cell system - suggest a negative regulatory role of PID as found for yeast transport data and for PID-PIN co-expression in tobacco (Fig. 5C).

Therefore, the eligible question that arises is what might be the molecular reason for these apparent discrepancies. One obvious option that is unfortunately hard to bypass is the fact that Arabidopsis PID competes with endogenous PID homologs in hetererologous systems (like yeast or tobacco). Second, a more likely and therefore relevant complication might be caused by opposite regulatory PID effects on ABCB and PIN activities themselves functionally interacting at least in certain tissues, that result depending on the test system (whole organs/single-cells) and its origin (tissue; root/shoot) in different net fluxes. Things are even more complicated by the fact that PIN-ABCB interactions have been shown to be synergistic (PIN1-ABCB1) and antagonistic (PIN2-ABCB1) (Blakeslee et al., 2007) and that PID was shown to have specific, dose-dependent and inverse regulatory roles in the root and shoot (Friml et al., 2004; Sukumar et al., 2009).
Third, many conclusions are based on studies employing kinase inhibitors, like staurosporine or quercetin, that have several partially overlapping functions and targets as well as relatively broad specificities. Although, our non-invasive measurements of IAA fluxes in the presence of protein kinase inhibitors support a positive PID regulation, many inhibitor studies should be interpreted with care. PIN1 relocation in *pin2* backgrounds by kinase inhibitors, like quercetin and chelerythrine, employs apparently transcriptional up-regulation (Santelia et al., 2008) that is PID-independent. Moreover, PID is handled as a negative regulator of auxin signaling (Christensen et al., 2000). Additionally, kinase inhibitors have been shown to interfere directly, kinase-independent with ABCBs (Goodfellow et al., 1996; Castro and Altenberg, 1997; Lee and Cho, 2006) that is not seen here in yeast but supported genetically via *rcn1 abcb1 abcb19* mutants (Mravec et al., 2008a).

In summary, our data suggest that PID, beside its function as a molecular switch of PIN polarity, has a direct impact on PIN- and ABCB-type auxin eflflux transporter activity. PINs and ABCBs are counter-regulated by PID in an action that is inhibited by binding of quercetin, an inhibitor of auxin transport and protein kinases. As alternative model, at the moment we cannot exclude that altered PIN polarity might be primarily the result of altered PIN/ABCB transport fluxes, which secondarily canalize PIN-cargoes to polar plasma membrane domains.
However, the fact that TWD1 itself is probably not an object of PID phosphorylation (not shown) suggests the attractive scenario where TWD1 might recruit PID for ABCB and/or PIN phosphorylation. PID is obviously not a direct target of NPA action that might be mediated by closely related kinases, like WAG1,2 that have been shown to share the regulation of identical NPA-sensitive PAT pathways (Santner and Watson, 2006) or other protein kinases, like co-purified putative TWD1 interactor, AHK5, that has been localized recently to cytoplasma and additionally to the plasma membrane (Desikan et al., 2008). PINs, and probably also ABCBs, would be dephoshorylated by trimeric PP2A-type phosphatases (Michniewicz et al., 2007; Sukumar et al., 2009) that show overlapping expression, locations and functions or by PP2A homologs, like the newly identified, putative TWD1 interactor PP2C (Fig. 1A).

Methods

Construction of TAP-TWD1 gain-of-function alleles

The TWD1 cDNA was inserted into pENTR-3C (Invitrogen) and transferred by Gateway recombination into pBIN20_NaTAP resulting in an N-terminal TAP fusion (35S:TAP-TWD1). pBIN20_NaTAP was constructed by transferring the 35S:NaTAP cassette from pNA-TAP (Invitrogen) into pBIN20. Columbia wild-type (At3g21640) was transformed with 35S:TAP-TWD1, positive transformants were selected by resistance to kanamycin and verified by Western analysis using anti-TAP and anti-TWD1. Homozygous lines were selected by progeny analysis and used for tandem affinity purification. For complemetation analysis, the *twd1-3* allele was complemented with 35S:TAP-TWD1 and analyzed phenotypically and biochemically for *twd1* complementation (not shown).

Tandem affinity purification of TAP-TWD1 interacting proteins

0.3 g of 5dag Arabidopsis roots from 35S:TAP-TWD1 or pBIN20_NaTAP vector control seedlings grown vertically on ½ MS plates at 8h light (100 uE) were homogenized with 0.3 ml lysis buffer (50mM Tris-HCl (pH8.0), 150mM NaCl, 1% Triton X-100) and the homogenate was centrifuged at 1,000g at 4°C for 5min. The supernatant was centrifuged at 8,000g at 4°C for 10min. and used for immunoprecipitation according to the method of *μ*MACS Epitope Tag Protein Isolation Kits (Miltenyi Biotec). Elutes were precipitated using 10% TCA/ acetone, pellets were dissolved in 6M Urea/ 100mM ammonium bicarbonate and digested with 0.01 mg/mL trypsin (sequence grade; Promega), 50 mM ammonium bicarbonate and incubated at 37 °C for 16 h.

Mass spectrometric analysis and database searching

LC-MS/MS analyses were performed by using an LTQ-Orbitrap XL-HTC-PAL system. Trypsin-digested peptides were loaded on the column (75 μm internal diameter, 15 cm; L-Column, CERI) using Paradigm MS4 HPLC pump (Michrom BioResources) and HTC-PAL autosampler (CTC analytics) and were eluted by a gradient of 5–45% (v/v) acetonitrile in 0.1% (v/v) formic acid over 70 min. The eluted peptides were introduced directly into LTQ-Orbitrap XL mass spectrometer with a flow rate of 300 nL/min and a spray voltage of 2.0 kV. The range of MS scan was *m/z* 450-1500 and the top three peaks were subjected to MS/MS analysis. MS/MS spectra were analyzed using the MASCOT server (version 2.2) searching the TAIR8 database (The Arabidopsis Information Resource). The mascot search parameters were as follows: set off the threshold at 0.05 in the ion-score cut off, peptide tolerance at 10 ppm, MS/MS tolerance at ± 0.8Da, peptide charge of 2+ or 3+, trypsin as enzyme allowing up to 1 missed cleavage, carboxymethylation on cysteines as a fixed modification and oxidation on methionine as a variable modification. Mascot identified vector control proteins were subtracted manually from TAP-TWD1 proteins, and proteins with a score above 30 were considered as significant interactors (Fig. 1 and Fig. S1).

Interaction analyses

PID-GST was expressed from pGEX4T-1-PID and purified as described in (Christensen et al., 2000). Ca 1 ug of PID-GST bound to glutathion sepharose was incubated with a 10-fold access of purified TWD1$^{1-337}$ protein (Bailly et al., 2008) and pull-down assays were performed as in (Geisler et al., 2003). Equal amounts of loading control, non-bound and elutes were separated by PAGE and detected using antisera, anti-TWD1$^{1075-1078}$. Anti-TWD1$^{1075-1078}$ were raised against two synthetic peptides covering amino acid 28-41 (anti-TWD1$^{1075-1076}$) and 56-70 (anti-TWD1$^{1077-1078}$). Peptides and each two polyclonal antisera in rabbits were produced by Operon (Tokyo, Japan).

PID and TWD1 cDNAs were inserted by PCR into pCR8-TOPO (Invitrogen) and transferred into compatible BRET destination vectors, pPZP222 and pBIN19 (Subramanian et al. 2006) by Gateway recombination. For BRET analysis, microsomes from *N. benthamiana* leaves co-infiltrated with agrobacteria (GV3101) containing 35S:PID-GFP and 35S:TWD1-rLuc (or corresponding empty vector controls) using standard protocols were prepared 4 dai. BRET signals were recorded from microsomes (each ca. 10ug) in the presence of 5μM coelenterazine (Biotium Inc.) and BRET ratios were calculated as described previously (Bailly et al., 2008). The results are the average of 10 readings collected every minute; presented are average values from 3 independent experiments (independent agrobaterium infiltrations) with each four replicas.

A**uxin transport assays**

ABCB1/PGP1, PIN1 and PIN2 were expressed from pNEV-PGP1, pAD4M-PIN1 and pADE1-PIN2 (Geisler et al. 2005, Balekslee 2007). PID DNA was PCR amplified from pGEX4T-1-PID (Christensen et al., 2000) and inserted BamHI/SalI into pRS314CUP, resulting in pRS314CUP-PID. The inactive pRS314CUP-MPID was constructed by introducing a D205A exchange by site-directed mutagenesis (QuikChange, Stratagene) in pRS314CUP-PID as described in (Christensen et al., 2000). Yeast IAA transport was assayed with the unspecific benzoic acid (BA) as control in parallel and performed as in (Bailly et al., 2008). Relative IAA/BA export is calculated from retained radioactivity as follows: (radioactivity in the yeast at time t=10 min.) - (radioactivity in the yeast at time t=0)) * (100%)/ (radioactivity in the yeast at t=0 min.).

IAA export from Arabidopsis mesophyl protoplasts was analyzed as in (Mravec et al., 2008b). IAA export from *N. benthamiana* mesophyll protoplasts was analyzed 4 dai agrobacterium-mediated co-transfection of ABCB1-MYC, PIN1 and PID:FLAG from pBI121-PGP1 (Sidler et al., 1998), pS001-PIN1 (Galweiler et al., 1998) and pMOA34-PID, respectively. 35S::PID-FLAG was constructed by subcloning the 35S:PID-FLAG-TERM cassette from pART7-PID-FLAG into binary plasmid pMO34. Protoplast preparation was identical except that a 25% percol gradient was used. Relative IAA/BA export is calculated from effluxed radioactivity as follows: ((radioactivity in the medium at time t) - (radioactivity in the medium at time t=0)) * (100%)/ (radioactivity in the medium at t=0).

Presented are average values from 6–8 independent experiments (yeast: independent transformations; protoplasts: infiltrations of independent agrobacterium transformants).

PID phosphorylation assays
PID-GST autophosphorylation and PID-dependent phosphorylation of myelin binding protein (MBP) was assayed as described in (Christensen et al., 2000). Assays were carried out in the presence of indicated drugs or solvent control and signal intensities of radiolabeled PID or MBP bands were quantified after PAGE using a Cyclone phosphoimager and Scion Image software 1.63 (Scion Corporate). Presented are average values from two independent PID-GST preparations with each 3-8 experiments.

Drug binding studies
Drug binding assays using *Arabidopsis* microsomes or PID-GST were performed as described elsewhere (Bailly, 2008b). Four replicates of each 20 μg of protein or 1 ug PID-GST were incubated with 10 nM radiolabelled drugs (30-60 Ci/mmol) in the presence and absence of the corresponding 10 μM non-radiolabelled drug. [^{3}H](G)quercetin (10 Ci/mmol; 1.0 mCi/ml) was custom-synthetized by ARC Inc.(St. Louis, USA) Reported values are the means of specific radiolabeled drug bound in the absence of cold drug (total) minus radiolabelled drug bound in the presence of cold drug (unspecific) from at least three independent experiments with four replicates each.

***In planta* analysis of IAA contents and transport**
Endogenous free IAA was quantified from shoot and root segments of MeOH extracted seedlings by using gas chromatography-mass spectrometry (GC-MS) as described in (Bouchard et al., 2006). Data are means of four independent lots of 30–50 seedlings each.
A platinum microelectrode was used to monitor IAA fluxes in *Arabidopsis* roots as described previously (Cox and Muday, 1994; Casimiro et al., 2003). For measurements, Columbia wild-type plants were grown in hydroponic cultures and used at 5 dag. Differential current from an IAA-selective microelectrode was recorded in the absence and presence of 5 μM NPA (taken from (Bailly et al., 2008), quercetin or chelerythrine.

Quantitative Analysis of Root Gravitropism
Root gravitropism in the dark of wild type and *pin1* (AT1G73590), *pin2* (AT5G57090), *abcb1/pgp1* (AT2G36910) and *abcb19/pgp19* (AT3G28860) mutant combinations (all ecotype Columbia (Col Wt) in the presence of protein kinase inhibitors (100nM quercetin, 400nM chelerythrine, 50nM staurosporine, 10 μM phorbole erster) was performed as described previously (Santelia et al., 2008). Helical wheels were plotted using PolarBar software.

Data Analysis
Data were analyzed using Prism 4.0b (GraphPad Software, San Diego, CA).

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Author contributions

S.H. and M.G. designed the research; S.H. performed auxin transport-, root gravitropism-, PIN1,2-GFP and ABCB1,19-GFP and drug binding- and PID phosphorylation analyses; B.W. analyzed BRET interaction; S.M. analyzed root IAA fluxes; S.P. analyzed free auxins; Y.F. performed TAP and MS analysis and M.G. performed PID-GST pulldown and drug binding and wrote the manuscript.

Abbreviations

IAA, indole-3-acetic acid; PAT, polar auxin transport; ABCB, ATP-binding cassette protein subfamily B; PIN, pin-formed; MDR, multidrug resistance; PGP, phosphoglycoprotein; PID, PINOID;

Figure Legends

Figure 1: PINOID physically interacts with the ABCB-TWD1 auxin efflux complex.
(A) TWD1 interactors identified by tandem affinity purification followed by shot-gun proteomics analysis using TAP-TWD1 as bait. Mascot identified vector control proteins were subtracted manually from TAP-TWD1 proteins, and proteins with a score above 30 were considered as significant interactors (see Table S1 for complete listing and Fig. S1 for peptide sequences).
(B) BRET analysis of microsomes prepared from *N. benthamiana* leaves co-transfected with TWD1-luciferase (TWD1-rLuc) and PID-YFP results in a positive BRET ratio suggesting physical interaction *in planta*.
(C) PID-GST immobilized to glutathion-sepharose is able to pull-down small amounts of purified $TWD1^{1-337}$ *in vitro* suggesting a weak but direct mode of interaction.

Figure 2: PID modulates ABCB- and PIN-mediated auxin efflux in yeast.
(A) PID specifically inhibits ABCB1-mediated IAA export while a mutated, inactive PID (MPID) or unrelated protein kinase BIN2 has no effect.
(B) PID has no significant influence on PIN2-mediated auxin export but specifically inhibits ABCB1/PIN1- and ABCB1/PIN2-mediated IAA export not seen with MPID. Note that PIN1 in the absence of ABCB1 is inactive in the yeast *S. cerevisiae* (Blakeslee et al., 2007).
Reduction of auxin retention (efflux) were calculated as relative export of initial export where ABCB1 was set to 100% (mean ± SE; n = 4-10).

Figure 3: The protein kinase and auxin transport inhibitor, quercetin, blocks PID-mediated ABCB1 inhibition.
Reduction of auxin retention (efflux) in the presence or absence of inhibitors (C, solvent control) was measured and is presented as relative export of initial export where ABCB1 solvent control was set to 100%, respectively (mean ± SE; n = 4-10). Note that chelerythrine addition led to activation of vector control (background) auxin efflux in the presence and absence of PID that required a relative presentation of activities.

Figure 4: Quercetin binding blocks PID kinase activity.
(A) *In vitro* auto-phosphorylation of PID-GST is inhibited by quercetin and chelerythrine while IAA and NPA have only slight effects. Coomassie stains (left panels) of non-phosphorylated PID (PID) was used as loading control. Autoradiographies (right panels) of auto-phosphorylated PID (PID-P), presumably represented by the upper band in the Coomassie stain (Christensen et al., 2000), were quantified and signal intensities were plottet against solvent controls (lower panel; means ± SE; n = 3).
(B) PID-GST but not GST alone binds quercetin and to a lesser amount as well IAA (mean ± SE; n = 4).
(C) Microsomes prepared from *PID* loss- and gain-of-function alleles show reduced and enhanced quercetin binding, respectively, but reciprocal NPA binding (mean ± SE; n = 4).

Figure 5: PID counter-actively regulates ABCB1- and PIN1-mediated auxin efflux *in planta*
(A) Auxin efflux from Arabidopsis *pid* protoplasts is strongly enhanced (means ± SE; n = 3).
(B) Free IAA levels are significantly elevated in the roots of gain-of-function and shoot of loss-of-function allels of *PID*, respectively. Data are mean ± SE (n = 4 with each 40-50 seedlings); absolute wild-type values were 0.58 ± 0.09
0.35 ± 0.02 pmol/mg (fresh weight) for roots and shoots, respectively. Significant differences (unpaired *t* test with Welch's correction, $p<0.05$) between wild-type and mutant alleles are indicated by asterisks.
(C) Co-transfection of *N. benthamiana* leaves with PID enhances ABCB1-mediated auxin efflux but strongly blocks PIN1 activity (mean ± SE; n = 4). Significant differences (unpaired *t* test with Welch's correction, $p<0.05$) between –PID controls are indicated by asterisks.
(D) Co-transfection of *N. benthamiana* leaves with PID-GFP does not significantly alter ABCB1- and PIN1-YFP expression and locations. Arrow marks a potential ER locations of PID-GFP in the presence of PIN1.

Figure 6: Protein kinase inhibitors, like quercetin and chelerythrin, block root PAT but rescue the *pin2* agravitropic phenotype.
(A) IAA influx profiles along wild type roots in the presence of inhibitors (5 μM) measured using an IAA-specific microelectrode. Positive fluxes represent a net IAA influx. Data are means ± S.E. (n = 12). Note that all treatments result in a reduced influx peak at 200 nm from the root tip that is shifted apically and basipetal by chelerythrine and quercetin, respectively, but not by NPA.
(B) Quercetin- but not chelerythrine-dependent rescue of *pin2* agravitropic root phenotype requires *PIN1*. The length of each bar represents the mean percentages ± S.D. of seedlings showing the same direction of root growth of at least three independent experiments; numbers correspond to the mean percent occurrence of 60 and 90° bending (sum of 60 and 90° sectors).

References

Abas, L., Benjamins, R., Malenica, N., Paciorek, T., Wisniewska, J., Moulinier-Anzola, J.C., Sieberer, T., Friml, J., and Luschnig, C. (2006). Intracellular trafficking and proteolysis of the Arabidopsis auxin-efflux facilitator PIN2 are involved in root gravitropism. Nat Cell Biol **8,** 249-256.

Abe, M., Fujiwara, M., Kurotani, K., Yokoi, S., and Shimamoto, K. (2008). Identification of dynamin as an interactor of rice GIGANTEA by tandem affinity purification (TAP). Plant Cell Physiol **49,** 420-432.

Bailly, A. (2008a). Cellular auxin transport and its regulation in Arabidopsis. PhD thesis, University of Zurich.

Bailly, A., Sovero, V., Vincenzetti, V., Santelia, D., Bartnik, D., Koenig, B.W., Mancuso, S., Martinoia, E., and Geisler, M. (2008). Modulation of P-glycoproteins by auxin transport inhibitors is mediated by interaction with immunophilins. J Biol Chem **283,** 21817-21826.

Bailly, A., Sovero, V. Vincenzetti, V., Santelia, D., Bartnik, D., Koenig, B.W., Mancuso, S., Martinoia, E. and Geisler, M. (2008b). Modulation of P-glycoproteins by auxin transport inhibitors is mediated by interaction with immunophilins. J. Biol. Chem. **283,** 21817-21826.

Benjamins, R., Quint, A., Weijers, D., Hooykaas, P., and Offringa, R. (2001). The PINOID protein kinase regulates organ development in Arabidopsis by enhancing polar auxin transport. Development **128,** 4057-4067.

Benjamins, R., Ampudia, C.S., Hooykaas, P.J., and Offringa, R. (2003). PINOID-mediated signaling involves calcium-binding proteins. Plant Physiol **132,** 1623-1630.

Bennett, S., Alvarez, J., Bossinger, G., and Smyth, D. (1995). Morphogenesis in pinoid mutants of Arabidopsis thaliana. Development **128,** 4057-4067.

Blakeslee, J.J., Bandyopadhyay, A., Lee, O.R., Mravec, J., Titapiwatanakun, B., Sauer, M., Makam, S.N., Cheng, Y., Bouchard, R., Adamec, J., Geisler, M., Nagashima, A., Sakai, T., Martinoia, E., Friml, J., Peer, W.A., and Murphy, A.S. (2007). Interactions among PIN-FORMED and P-glycoprotein auxin transporters in Arabidopsis. Plant Cell **19,** 131-147.

Blilou, I., Xu, J., Wildwater, M., Willemsen, V., Paponov, I., Friml, J., Heidstra, R., Aida, M., Palme, K., and Scheres, B. (2005). The PIN auxin efflux facilitator network controls growth and patterning in Arabidopsis roots. Nature **433,** 39-44.

Bouchard, R., Bailly, A., Blakeslee, J.J., Oehring, S.C., Vincenzetti, V., Lee, O.R., Paponov, I., Palme, K., Mancuso, S., Murphy, A.S., Schulz, B., and Geisler, M. (2006). Immunophilin-like TWISTED DWARF1 modulates auxin efflux activities of Arabidopsis P-glycoproteins. J Biol Chem **281,** 30603-30612.

Casimiro, I., Beeckman, T., Graham, N., Bhalerao, R., Zhang, H.M., Casero, P., Sandberg, G., and Bennett, M.J. (2003). Dissecting Arabidopsis lateral root development. Trends in Plant Science **8,** 165-171.

Castro, A.F., and Altenberg, G.A. (1997). Inhibition of drug transport by genistein in multidrug-resistant cells expressing P-glycoprotein. Biochem Pharmacol **53,** 89-93.

Castro, A.F., Horton, J.K., Vanoye, C.G., and Altenberg, G.A. (1999). Mechanism of inhibition of P-glycoprotein-mediated drug transport by protein kinase C blockers. Biochem Pharmacol **58,** 1723-1733.

Christensen, S.K., Dagenais, N., Chory, J., and Weigel, D. (2000). Regulation of auxin response by the protein kinase PINOID. Cell **100,** 469-478.

Conseil, G., Perez-Victoria, J.M., Jault, J.M., Gamarro, F., Goffeau, A., Hofmann, J., and Di Pietro, A. (2001). Protein kinase C effectors bind to multidrug ABC transporters and inhibit their activity. Biochemistry **40,** 2564-2571.

Cox, D.N., and Muday, G.K. (1994). NPA binding activity is peripheral to the plasma membrane and is associated with the cytoskeleton. Plant Cell **6,** 1941-1953.

Delbarre, A., Muller, P., and Guern, J. (1998). Short-Lived and Phosphorylated Proteins Contribute to Carrier-Mediated Efflux, but Not to Influx, of Auxin in Suspension-Cultured Tobacco Cells. Plant Physiol **116,** 833-844.

Deruere, J., Jackson, K., Garbers, C., Soll, D., and Delong, A. (1999). The RCN1-encoded A subunit of protein phosphatase 2A increases phosphatase activity in vivo. Plant J **20,** 389-399.

Desikan, R., Horak, J., Chaban, C., Mira-Rodado, V., Witthoft, J., Elgass, K., Grefen, C., Cheung, M.K., Meixner, A.J., Hooley, R., Neill, S.J., Hancock, J.T., and Harter, K. (2008). The histidine kinase AHK5 integrates endogenous and environmental signals in Arabidopsis guard cells. PLoS One **3,** e2491.

Edlich, F., Maestre-Martinez, M., Jarczowski, F., Weiwad, M., Moutty, M.C., Malesevic, M., Jahreis, G., Fischer, G., and Lucke, C. (2007). A novel calmodulin-Ca2+ target recognition activates the Bcl-2 regulator FKBP38. J Biol Chem **282,** 36496-36504.

Friml, J., Yang, X., Michniewicz, M., Weijers, D., Quint, A., Tietz, O., Benjamins, R., Ouwerkerk, P.B., Ljung, K., Sandberg, G., Hooykaas, P.J., Palme, K., and Offringa, R. (2004). A PINOID-dependent binary switch in apical-basal PIN polar targeting directs auxin efflux. Science **306,** 862-865.

Furutani, M., Kajiwara, T., Kato, T., Treml, B.S., Stockum, C., Torres-Ruiz, R.A., and Tasaka, M. (2007). The gene MACCHI-BOU 4/ENHANCER OF PINOID encodes a NPH3-like protein and reveals similarities between organogenesis and phototropism at the molecular level. Development **134,** 3849-3859.

Galvan-Ampudia, C.S., and Offringa, R. (2007). Plant evolution: AGC kinases tell the auxin tale. Trends Plant Sci **12,** 541-547.

Galweiler, L., Guan, C.H., Muller, A., Wisman, E., Mendgen, K., Yephremov, A., and Palme, K. (1998). Regulation of polar auxin transport by AtPIN1 in Arabidopsis vascular tissue. Science **282,** 2226-2230.

Garbers, C., DeLong, A., Deruere, J., Bernasconi, P., and Soll, D. (1996). A mutation in protein phosphatase 2A regulatory subunit A affects auxin transport in Arabidopsis. EMBO J **15,** 2115-2124.

Geisler, M., and Bailly, A. (2007). Tete-a-tete: the function of FKBPs in plant development. Trends Plant Sci **12,** 465-473.

Geisler, M., Kolukisaoglu, H.U., Bouchard, R., Billion, K., Berger, J., Saal, B., Frangne, N., Koncz-Kalman, Z., Koncz, C., Dudler, R., Blakeslee, J.J., Murphy, A.S., Martinoia, E., and Schulz, B. (2003). TWISTED DWARF1, a unique plasma membrane-anchored immunophilin-like protein, interacts with Arabidopsis multidrug resistance-like transporters AtPGP1 and AtPGP19. Mol Biol Cell **14,** 4238-4249.

Geisler, M., Blakeslee, J.J., Bouchard, R., Lee, O.R., Vincenzetti, V., Bandyopadhyay, A., Titapiwatanakun, B., Peer, W.A., Bailly, A., Richards, E.L., Ejendal, K.F., Smith, A.P., Baroux, C., Grossniklaus, U., Muller, A., Hrycyna, C.A., Dudler, R., Murphy, A.S., and Martinoia, E. (2005). Cellular efflux of auxin catalyzed by the Arabidopsis MDR/PGP transporter AtPGP1. Plant J **44,** 179-194.

Goodfellow, H.R., Sardini, A., Ruetz, S., Callaghan, R., Gros, P., McNaughton, P.A., and Higgins, C.F. (1996). Protein kinase C-mediated phosphorylation does not regulate drug transport by the human multidrug resistance P-glycoprotein. J Biol Chem **271,** 13668-13674.

Graziani, Y., Erikson, E., and Erikson, R.L. (1983). The effect of quercetin on the phosphorylation activity of the Rous sarcoma virus transforming gene product in vitro and in vivo. Eur J Biochem **135,** 583-589.

Graziani, Y., Chayoth, R., Karny, N., Feldman, B., and Levy, J. (1982). Regulation of protein kinases activity by quercetin in Ehrlich ascites tumor cells. Biochim Biophys Acta **714,** 415-421.

Gschwendt, M., Horn, F., Kittstein, W., and Marks, F. (1983). Inhibition of the calcium- and phospholipid-dependent protein kinase activity from mouse brain cytosol by quercetin. Biochem Biophys Res Commun **117,** 444-447.

Herbert, J.M., Augereau, J.M., Gleye, J., and Maffrand, J.P. (1990). Chelerythrine is a potent and specific inhibitor of protein kinase C. Biochem Biophys Res Commun **172,** 993-999.

Idriss, H.T., Hannun, Y.A., Boulpaep, E., and Basavappa, S. (2000). Regulation of volume-activated chloride channels by P-glycoprotein: phosphorylation has the final say! J Physiol **524 Pt 3,** 629-636.

Iwama, A., Yamashino, T., Tanaka, Y., Sakakibara, H., Kakimoto, T., Sato, S., Kato, T., Tabata, S., Nagatani, A., and Mizuno, T. (2007). AHK5 histidine kinase regulates root elongation through an ETR1-dependent abscisic acid and ethylene signaling pathway in Arabidopsis thaliana. Plant Cell Physiol **48,** 375-380.

Kamphausen, T., Fanghanel, J., Neumann, D., Schulz, B., and Rahfeld, J.U. (2002). Characterization of Arabidopsis thaliana AtFKBP42 that is membrane-bound and interacts with Hsp90. Plant J **32,** 263-276.

Lee, S.H., and Cho, H.T. (2006). PINOID positively regulates auxin efflux in Arabidopsis root hair cells and tobacco cells. Plant Cell **18,** 1604-1616.

Li, J., and Nam, K.H. (2002). Regulation of brassinosteroid signaling by a GSK3/SHAGGY-like kinase. Science **295,** 1299-1301.

Li, J., Nam, K.H., Vafeados, D., and Chory, J. (2001). BIN2, a new brassinosteroid-insensitive locus in Arabidopsis. Plant Physiol **127,** 14-22.

Limtrakul, P., Khantamat, O., and Pintha, K. (2005). Inhibition of P-glycoprotein function and expression by kaempferol and quercetin. J Chemother **17,** 86-95.

Lomax, T.L., Muday, G.K., and Rubery, P.H. (1995). Auxin transport. In Plant Hormones: Physiology, Biochemistry and Molecular Biology, P.J. Davies, ed (Dordrecht, Netherlands: Kluwer), pp. 509-530.

Mancuso, S., Marras, A.M., Magnus, V., and Baluska, F. (2005). Noninvasive and continuous recordings of auxin fluxes in intact root apex with a carbon nanotube-modified and self-referencing microelectrode. Anal Biochem **341,** 344-351.

Michniewicz, M., Zago, M.K., Abas, L., Weijers, D., Schweighofer, A., Meskiene, I., Heisler, M.G., Ohno, C., Zhang, J., Huang, F., Schwab, R., Weigel, D., Meyerowitz, E.M., Luschnig, C., Offringa, R., and Friml, J. (2007). Antagonistic regulation of PIN phosphorylation by PP2A and PINOID directs auxin flux. Cell **130,** 1044-1056.

Mravec, J., Kubes, M., Bielach, A., Gaykova, V., Petrasek, J., Skupa, P., Chand, S., Benkova, E., Zazimalova, E., and Friml, J. (2008a). Interaction of PIN and PGP transport mechanisms in auxin distribution-dependent development. Development **135,** 3345-3354.

Mravec, J., Bailly, A., Hoyerová, K., Skůpa, P., Petrášek, J., Gaykova, V., Stierhof, Y.-D., Luschnig, C., Zažímalová, E., Geisler, M., and Friml, J. (2008b). ER-localized PIN5 auxin transporter mediates cellular auxin homeostasis. Nature (submitted).

Muday, G.K., and DeLong, A. (2001). Polar auxin transport: controlling where and how much. Trends Plant Sci **6,** 535-542.

Nagashima, A., Suzuki, G., Uehara, Y., Saji, K., Furukawa, T., Koshiba, T., Sekimoto, M., Fujioka, S., Kuroha, T., Kojima, M., Sakakibara, H., Fujisawa, N., Okada, K., and Sakai, T. (2008). Phytochromes and cryptochromes regulate the differential growth of Arabidopsis hypocotyls in both a PGP19-dependent and a PGP19-independent manner. Plant J **53,** 516-529.

Nuhse, T.S., Stensballe, A., Jensen, O.N., and Peck, S.C. (2004). Phosphoproteomics of the Arabidopsis plasma membrane and a new phosphorylation site database. Plant Cell **16,** 2394-2405.

Okamoto, Tanaka, and Sakai. (1991). Molecular cloning and analysis of the c DNA for an auxin-regulated calmodulin gene. Plant Cell Physiol. **36,** 1531-1539.

Palme, K., and Galweiler, L. (1999). PIN-pointing the molecular basis of auxin transport. Curr Opin Plant Biol **2,** 375-381.

Peer, W.A., and Murphy, A.S. (2007). Flavonoids and auxin transport: modulators or regulators? Trends Plant Sci **12,** 556-563.

Petrasek, J., and Friml, J. (2009). Auxin transport routes in plant development. Development **136,** 2675-2688.

Pu, S., Vlasblom, J., Emili, A., Greenblatt, J., and Wodak, S.J. (2007). Identifying functional modules in the physical interactome of Saccharomyces cerevisiae. Proteomics **7,** 944-960.

Rashotte, A.M., DeLong, A., and Muday, G.K. (2001). Genetic and chemical reductions in protein phosphatase activity alter auxin transport, gravity response, and lateral root growth. Plant Cell **13,** 1683-1697.

Rojas-Pierce, M., Titapiwatanakun, B., Sohn, E.J., Fang, F., Larive, C.K., Blakeslee, J., Cheng, Y., Cutler, S.R., Peer, W.A., Murphy, A.S., and Raikhel, N.V. (2007). Arabidopsis P-glycoprotein19 participates in the inhibition of gravitropism by gravacin. Chem Biol **14,** 1366-1376.

Santelia, D., Vincenzetti, V., Azzarello, E., Bovet, L., Fukao, Y., Duchtig, P., Mancuso, S., Martinoia, E., and Geisler, M. (2005). MDR-like ABC transporter AtPGP4 is involved in auxin-mediated lateral root and root hair development. FEBS Lett **579,** 5399-5406.

Santelia, D., Henrichs, S., Vincenzetti, V., Sauer, M., Bigler, L., Klein, M., Bailly, A., Lee, Y., Friml, J., Geisler, M., and Martinoia, E. (2008). Flavonoids Redirect PIN-mediated Polar Auxin Fluxes during Root Gravitropic Responses. J Biol Chem **283,** 31218-31226.

Santner, A.A., and Watson, J.C. (2006). The WAG1 and WAG2 protein kinases negatively regulate root waving in Arabidopsis. Plant J **45,** 752-764.

Schweighofer, A., Kazanaviciute, V., Scheikl, E., Teige, M., Doczi, R., Hirt, H., Schwanninger, M., Kant, M., Schuurink, R., Mauch, F., Buchala, A., Cardinale, F., and Meskiene, I. (2007). The PP2C-type phosphatase AP2C1, which negatively regulates MPK4 and MPK6, modulates innate immunity, jasmonic acid, and ethylene levels in Arabidopsis. Plant Cell **19,** 2213-2224.

Sidler, M., Hassa, P., Hasan, S., Ringli, C., and Dudler, R. (1998). Involvement of an ABC transporter in a developmental pathway regulating hypocotyl cell elongation in the light. Plant Cell **10,** 1623-1636.

Sistrunk, M.L., Antosiewicz, D.M., Purugganan, M.M., and Braam, J. (1994). Arabidopsis TCH3 encodes a novel Ca2+ binding protein and shows environmentally induced and tissue-specific regulation. Plant Cell **6,** 1553-1565.

Sukumar, P., Edwards, K.S., Rahman, A., Delong, A., and Muday, G.K. (2009). PINOID kinase regulates root gravitropism through modulation of PIN2-dependent basipetal auxin transport in Arabidopsis. Plant Physiol **150,** 722-735.

Terasaka, K., Blakeslee, J.J., Titapiwatanakun, B., Peer, W.A., Bandyopadhyay, A., Makam, S.N., Lee, O.R., Richards, E.L., Murphy, A.S., Sato, F., and Yazaki, K. (2005). PGP4, an ATP binding cassette P-glycoprotein, catalyzes auxin transport in Arabidopsis thaliana roots. Plant Cell **17,** 2922-2939.

Van Leene, J., Witters, E., Inze, D., and De Jaeger, G. (2008). Boosting tandem affinity purification of plant protein complexes. Trends Plant Sci **13,** 517-520.

Vanneste, S., and Friml, J. (2009). Auxin: a trigger for change in plant development. Cell **136,** 1005-1016.

Vert, G., Walcher, C.L., Chory, J., and Nemhauser, J.L. (2008). Integration of auxin and brassinosteroid pathways by Auxin Response Factor 2. Proc Natl Acad Sci U S A **105,** 9829-9834.

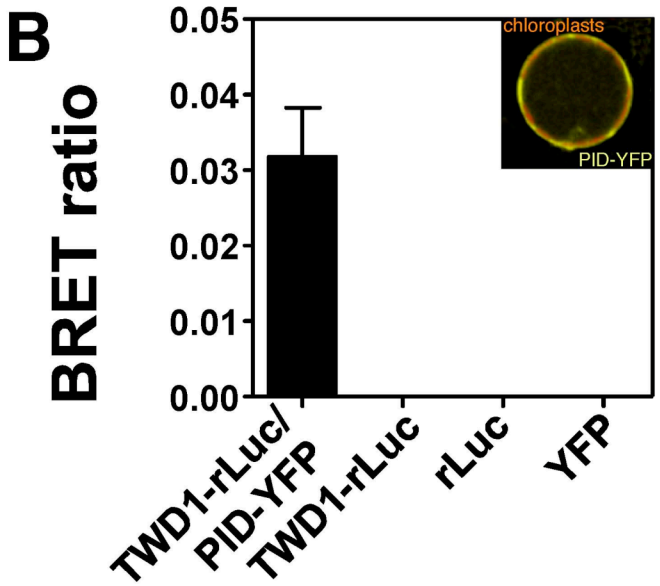
Vieten, A., Sauer, M., Brewer, P.B., and Friml, J. (2007). Molecular and cellular aspects of auxin-transport-mediated development. Trends Plant Sci **12,** 160-168.

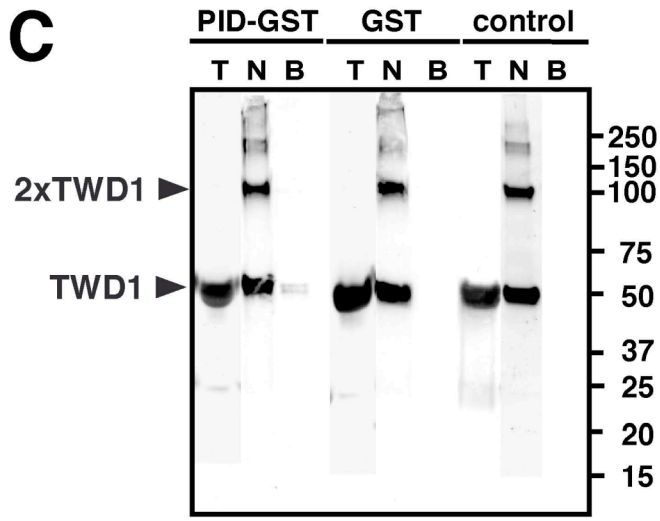
Wisniewska, J., Xu, J., Seifertova, D., Brewer, P.B., Ruzicka, K., Blilou, I., Rouquie, D., Benkova, E., Scheres, B., and Friml, J. (2006). Polar PIN localization directs auxin flow in plants. Science **312,** 883.

Yang, H., and Murphy, A.S. (2009). Functional expression and characterization of Arabidopsis ABCB, AUX 1 and PIN auxin transporters in Schizosaccharomyces pombe. Plant J **59,** 179-191.

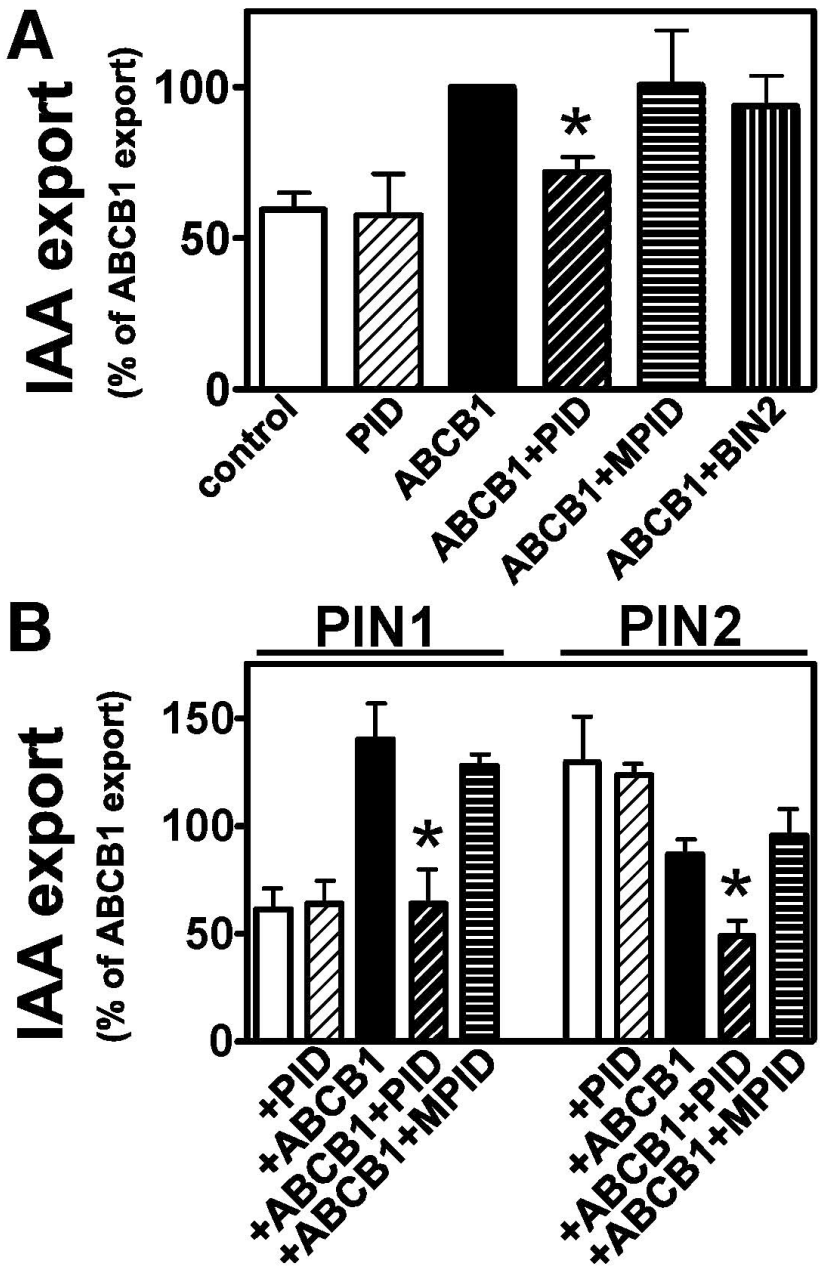
A

Protein	AGI	score	cover (%)
TWD1 (TWISTED DWARF 1)	*AT3G21640*	*129*	*13.4*
polynucleotide adenylyltransferase family protein	AT3G48830	61	3.2
similar to unknown protein	AT4G25920	38	1.1
AHK5 (CYTOKININ INDEPENDENT 2)	AT5G10720	34	1.2
PID (PINOID)	*AT2G34650*	*32*	*3.0*
protein phosphatase 2C, putative	AT2G30020	32	2.5





Henrichs et al. Fig. 1



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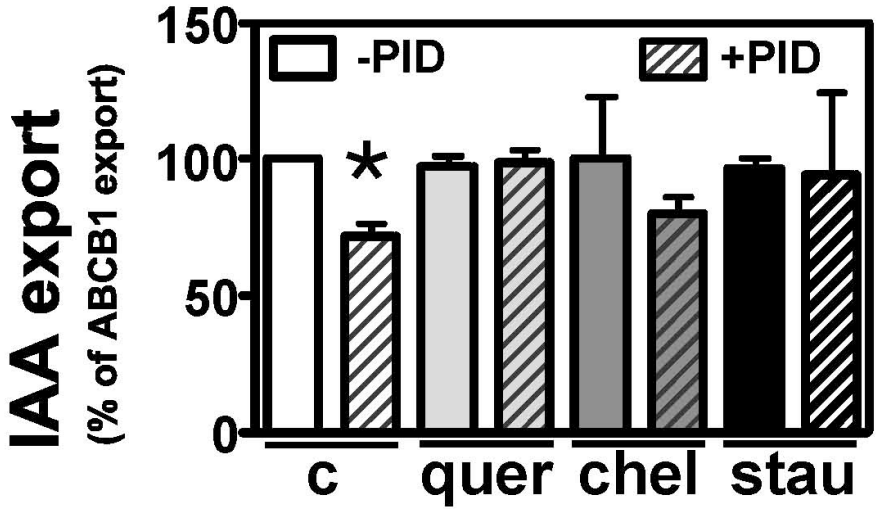

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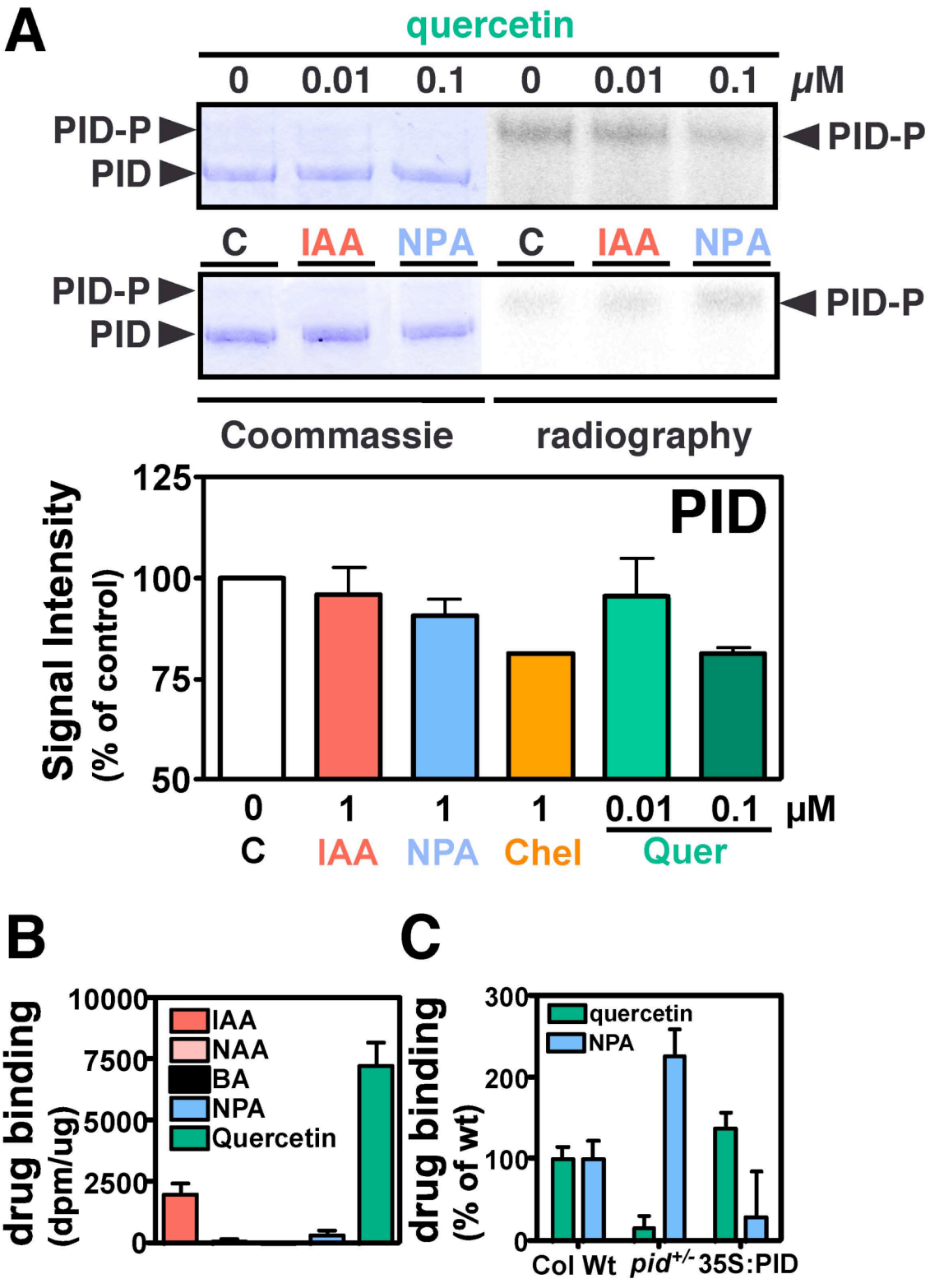

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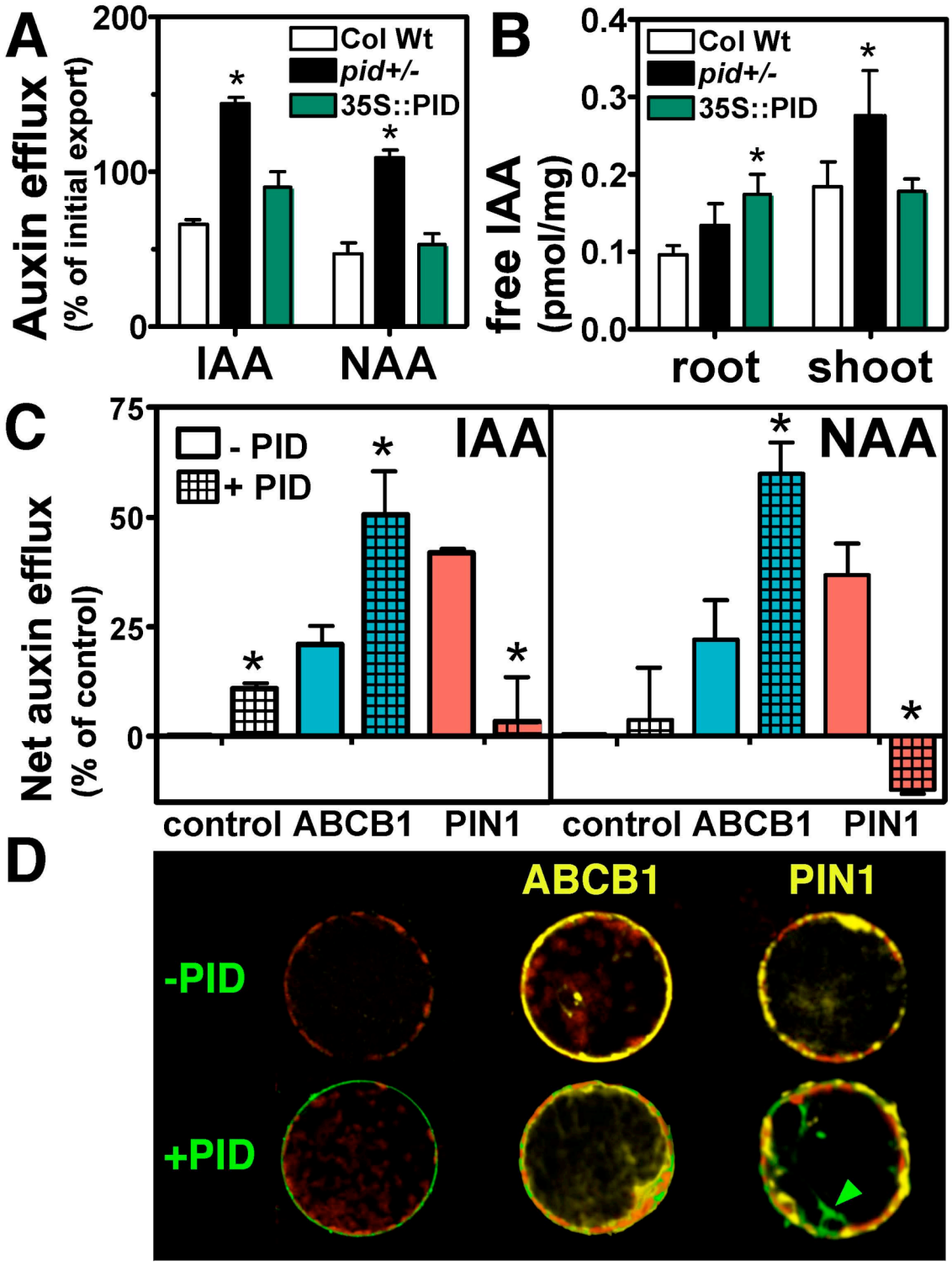
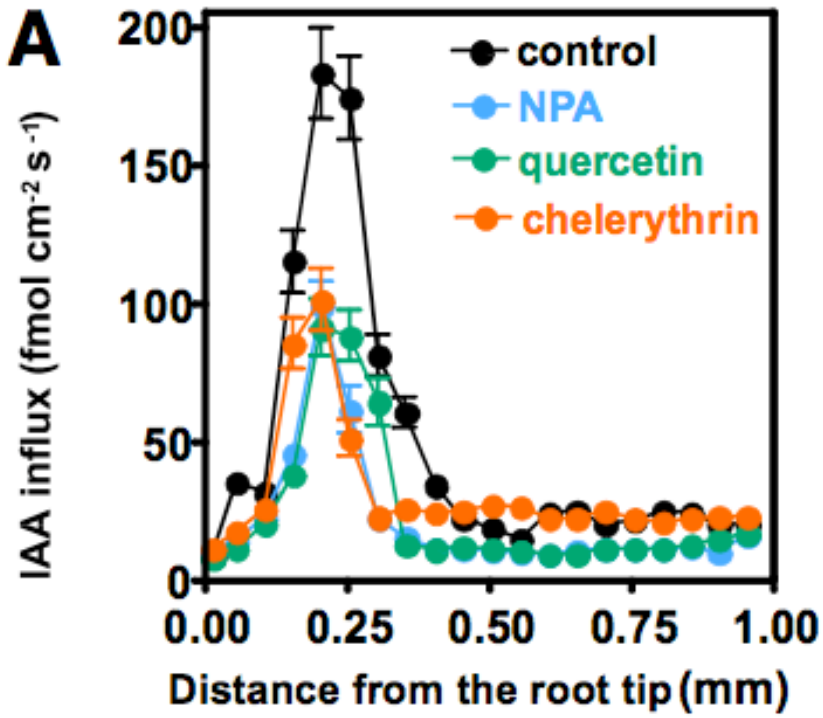
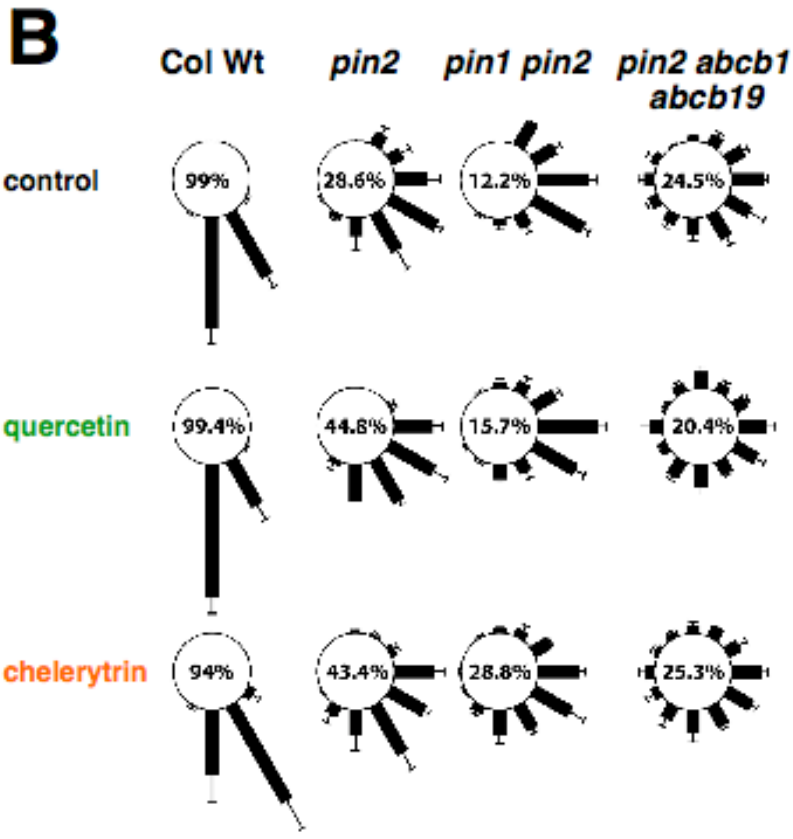


Henrichs et al. Fig. 5





Henrichs et al. Fig. 6

Supplemental data

The AGC kinase PINOID mediates quercetin-dependent, counter-regulation of ABCB- and PIN-catalyzed auxin transport

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Supplementary Figure Legends

Table S1: TWD1 interactors identified by tandem affinity purification followed by shotgun proteomic analysis using TAP-TWD1 as bait.

Mascot identified vector control proteins and TAP-TWD1 proteins with the AGI codes (prot_acc), protein scores (prot_score) and protein cover (prot_cover). Proteins with a score above 30 that are not found in vector controls are colored in yellow and summarized in Fig. 1A).

Figure S1: TAP-purified protein separation and peptide sequences of TWD1 and PID

(A) Silver-stain of TAP-purified vector control and TAP-TWD1 proteins after PAGE.

(B) Protein sequences of identified TWD1 and PID peptides with protein coverage.

Figure S2: PID modulation of ABCB- and PIN-mediated IAA export is specific.

(A) Neither, a mutated, inactive PID (MPID) nor an unrelated protein kinase, BIN2, does inhibit ABCB1-mediated benzoic acid (BA) export.

(B) PID and MPID have no significant influence on PIN1, PIN2, ABCB1/PIN1- or ABCB1/PIN2-mediated IAA export.

Reduction of BA retention (efflux) was calculated as relative export of initial export where ABCB1 was set to 100% (mean ± SE; n = 4-10).

(C) Co-expression with PID or MPID does not significantly alter expression or location of ABCB1-YFP as shown by confocal microscopy analysis.

Figure S3: Quercetin as well as mammalian protein kinase inhibitors, chelerythrine and staurosporin, have no significant effect on ABCB1 mediated BA export both in the absence and presence of PID.

Reduction of auxin retention (efflux) were calculated as relative export of initial export where ABCB1 solvent control was set to 100%, respectively (mean ± SE; n = 4-10).

Figure S4: Quercetin binding blocks trans-phosphorylation of MBP by PID

Inhibition of *in vitro* autophosphorylation of PID-GST by quercetin correlates with inhibition of MBP trans-phosphorylation. In contrast to autophosporylation, chelerythrine has only a slight effect on MBP phosphorylation. Coomassie stains (left panels) of non-phosphorylated PID (PID) was used as loading control. Autoradiographies (right panels) of autophosphorylated PID (PID-P), presumably represented by the upper band in the Coomassie stain (Christensen et al., 2000), were quantified and signal intensities were plottet against solvent controls (lower panel; means ± SE; n = 3).

Figure S5: Time-course of auxin efflux from *PID* gain- and loss of function protoplasts.

IAA (left) and NAA efflux (right) from Arabidopsis *pid* protoplasts is strongly enhanced while *PID* gain-of function has only a mild, non-significant effect (means ± SE; n = 3).

Figure S6: Staurosporine and phorbole ester do not rescue the *pin2* agravitropic phenotype.

Root bending analysis of after 12h kinase inhibitor treatment (100nM quercetin, 400nM chelerythrine, 50nM staurosporine, 10 μM phorbole ester). The length of each bar represents the mean percentages ± S.D. of seedlings showing the same direction of root growth of at least three independent experiments; numbers correspond to the mean percent occurrence of 60 and 90° bending (sum of 60 and 90° sectors).

Figure S7: Protein kinase inhibitors do not significantly alter ABCB1-, ABCB19, PIN1- and PIN2 root locations.

Arrows mark ectopic PIN1 expression at epidermal and cortical *PIN2* locations caused by quercetin and chelerythrine, enhanced ABCB1 expression in Columella cells upon chelerythrine treatments, as well as slightly elevated ABCB19 expression in the stele by quercetin and chelerythrine.

Henrichs et al. Table S1

prot_acc	Identifeid Proteins in vector control	prot_score	prot_cover
AT3G09260	PYK10 (phosphate starvation-response 3.1); hydrolase, hy	2040	55.5
AT3G15730	PLDALPHA1 (PHOSPHOLIPASE D ALPHA 1); phospholipa	895	43.3
AT3G16420	PBP1 (PYK10-BINDING PROTEIN 1)	519	55.4
AT1G66280	glycosyl hydrolase family 1 protein	355	12.6
AT3G16460	jacalin lectin family protein	323	23.3
AT3G16470	JR1 (Jacalin lectin family protein)	178	25.1
AT3G01670	similar to unknown protein [Arabidopsis thaliana] (TAIR:AT	171	13.7
AT1G52400	BGL1 (BETA-GLUCOSIDASE HOMOLOG 1); hydrolase, hy	169	13.6
AT3G14210	ESM1 (EPITHIOSPECIFIER MODIFIER 1); carboxylesterase	159	15.8
ATCG00490	large subunit of RUBISCO.	158	16.3
AT1G52570	PLDALPHA2 (PHOSPHLIPASE D ALPHA 2); phospholipas	153	6.9
AT1G54010	myrosinase-associated protein, putative	129	20.5
AT5G26280	meprin and TRAF homology domain-containing protein / M	81	8.9
AT4G33630	EX1 (EXECUTER1)	81	0.7
AT3G20370	meprin and TRAF homology domain-containing protein / M	71	7.7
AT3G16450	jacalin lectin family protein	53	13.7
AT3G01680	similar to unknown protein [Arabidopsis thaliana] (TAIR:AT	51	6.4
AT4G24750	similar to rhodanese-like domain-containing protein [Arabi	47	2.1
AT1G54000	myrosinase-associated protein, putative	37	9
AT1G58270	ZW9	37	3.8
AT2G27120	POL2B/TIL2 (TILTED2); DNA-directed DNA polymerase	35	0.2
AT2G47300	ribonuclease P	35	0.6
AT1G29120	similar to unknown protein [Arabidopsis thaliana] (TAIR:AT	32	1.1
AT1G50030	TOR (TARGET OF RAPAMYCIN)	32	0.2
AT1G80450	VQ motif-containing protein	32	2.8
AT2G20240	similar to unknown protein [Arabidopsis thaliana] (TAIR:AT	32	0.8
AT1G06170	basic helix-loop-helix (bHLH) family protein	32	2.6
AT4G01650	similar to unknown protein [Arabidopsis thaliana] (TAIR:AT	31	5.9
AT1G52000	jacalin lectin family protein	30	1.8
AT2G33040	ATP synthase gamma chain, mitochondrial (ATPC)	29	2.8
AT1G32375	F-box family protein	28	2.6
AT3G50820	PSBO-2/PSBO2 (PHOTOSYSTEM II SUBUNIT O-2); oxyg	28	10.6
AT1G48370	YSL8 (YELLOW STRIPE LIKE 8); oligopeptide transporter	27	1.5
AT3G16940	calmodulin-binding protein	26	1.2
AT4G25920	similar to unknown protein [Arabidopsis thaliana] (TAIR:AT	26	4.1
AT1G01360	similar to unknown protein [Arabidopsis thaliana] (TAIR:AT	25	2.7
AT3G62110	glycoside hydrolase family 28 protein / polygalacturonase (	25	1.1
AT5G38750	asparaginyl-tRNA synthetase family	25	4.4
AT5G28910	similar to unknown protein [Arabidopsis thaliana] (TAIR:AT	25	2.2
AT5G19950	similar to unknown protein [Arabidopsis thaliana] (TAIR:AT	24	4.1

Henrichs et al. Table S1

AT2G36240	binding	24	5
AT1G64530	RWP-RK domain-containing protein	23	2
AT1G53880	GTP binding / translation initiation factor	23	1.7
AT2G25980	jacalin lectin family protein	22	3.1
AT2G23390	similar to hypothetical protein OsI_015489 [Oryza sativa (ir	22	1.1
AT2G35110	GRL/NAP1/NAPP (NCK-ASSOCIATED PROTEIN); transc	22	1.4
AT1G50140	ATPase	22	0.5
AT3G05050	protein kinase family protein	22	0.8
AT1G09440	protein kinase family protein	21	2.1
AT5G53670	similar to hypothetical protein [Vitis vinifera] (GB:CAN812	21	2.9

Henrichs et al. Table S1

prot_acc	Identified Proteins in TWD1-TAP	prot_score	prot_cover
AT3G09260	PYK10 (phosphate starvation-response 3.1); hydrola	1813	50
AT3G16420	PBP1 (PYK10-BINDING PROTEIN 1)	840	60.1
AT3G16460	jacalin lectin family protein	614	32.6
AT3G15730	PLDALPHA1 (PHOSPHOLIPASE D ALPHA 1); phos	330	26.9
AT1G66280	glycosyl hydrolase family 1 protein	308	12.6
ATCG00490	large subunit of RUBISCO.	283	21.3
AT1G54010	myrosinase-associated protein, putative	223	28.5
AT3G21640	TWD1 (TWISTED DWARF 1); FK506 binding / peptid	129	6
AT3G14210	ESM1 (EPITHIOSPECIFIER MODIFIER 1); carboxyle	122	24.5
AT3G16450	jacalin lectin family protein	120	41
AT2G39730	RCA (RUBISCO ACTIVASE)	93	3.2
AT4G33630	EX1 (EXECUTER1)	86	0.7
AT3G16470	JR1 (Jacalin lectin family protein)	72	10.9
AT3G48830	polynucleotide adenylyltransferase family protein / I	61	1.1
AT4G24750	similar to rhodanese-like domain-containing protein	44	2.1
AT1G54000	myrosinase-associated protein, putative	43	7.9
AT5G26280	meprin and TRAF homology domain-containing prot	41	16.9
AT1G58270	ZW9	40	3.8
AT1G80450	VQ motif-containing protein	39	2.8
AT2G27120	POL2B/TIL2 (TILTED2); DNA-directed DNA polyme	39	0.2
AT2G47300	ribonuclease P	39	0.6
AT3G20370	meprin and TRAF homology domain-containing prot	38	7.1
AT4G25920	similar to unknown protein [Arabidopsis thaliana] (T	38	4.1
AT1G52570	PLDALPHA2 (PHOSPHLIPASE D ALPHA 2); phosph	35	4.6
AT5G10720	AHK5 (CYTOKININ INDEPENDENT 2)	34	1.2
AT2G25980	jacalin lectin family protein	33	3.3
AT2G34650	PID (PINOID); kinase	32	3
AT2G30020	protein phosphatase 2C, putative / PP2C, putative	32	2.5
AT3G14270	phosphatidylinositol-4-phosphate 5-kinase family pr	30	0.4
AT1G01360	similar to unknown protein [Arabidopsis thaliana] (T	30	2.7
AT3G62110	glycoside hydrolase family 28 protein / polygalactur	30	1.1
AT5G28910	similar to unknown protein [Arabidopsis thaliana] (T	30	2.2
AT1G33240	AT-GTL1 (Arabidopsis thaliana GT2-like 1); transcri	30	1.2
AT4G37460	binding	29	0.8
AT1G47765	F-box family protein	28	1.6
AT3G54230	nucleic acid binding	28	0.8
AT2G18850	similar to SET domain-containing protein [Arabidops	28	4.2
AT2G39190	ATATH8 (ABC2 homolog 8)	28	2.2
AT4G01650	similar to unknown protein [Arabidopsis thaliana] (T	27	5.9
AT1G70840	MLP31 (MLP-LIKE PROTEIN 31)	27	11.1

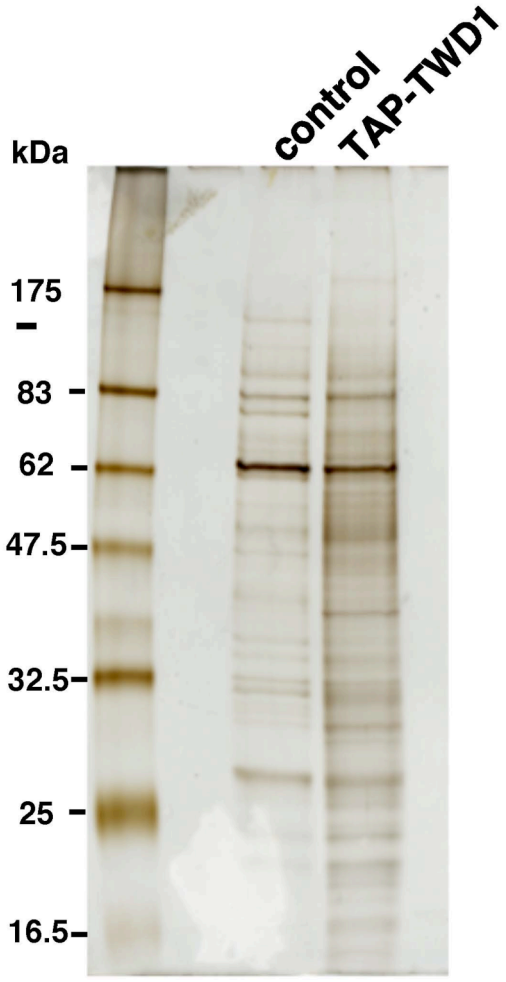
Henrichs et al. Table S1

AT1G27190	leucine-rich repeat transmembrane protein kinase, p	26	3.8
AT2G29480	ATGSTU2 (GLUTATHIONE S-TRANSFERASE 20); (	26	5.8
AT5G61280	remorin family protein	26	1.9
AT2G27170	TTN7 (TITAN7); ATP binding	25	1.1
AT1G61010	CPSF73-I; protein binding	25	1.3
AT1G06470	phosphate translocator-related	24	2.4
AT4G00750	dehydration-responsive family protein	24	1.7
AT4G38825	similar to auxin-responsive protein, putative [Arabid	24	12.4
AT1G49240	ACT8 (ACTIN 8); structural constituent of cytoskele	24	3.2
AT5G43020	leucine-rich repeat transmembrane protein kinase, p	24	2.7
AT2G33040	ATP synthase gamma chain, mitochondrial (ATPC)	23	2.8
AT3G45610	Dof-type zinc finger domain-containing protein	23	3.7
AT1G71390	disease resistance family protein / LRR family prote	23	1.7
AT5G53670	similar to hypothetical protein [Vitis vinifera] (GB:CA	23	2.9
AT5G67100	ICU2 (INCURVATA2); DNA-directed DNA polymerase	23	1.4
AT2G23390	similar to hypothetical protein OsI_015489 [Oryza sa	23	1.1
AT3G01680	similar to unknown protein [Arabidopsis thaliana] (TA	22	1.5
AT2G36740	ATSWC2/SWC2; DNA binding	22	6.3
AT4G14310	similar to hypothetical protein OsI_009189 [Oryza sa	21	2.1
AT4G14385	similar to unnamed protein product [Vitis vinifera] ((	21	12.9

A

	TWD1	PID
Peptide	ALYQK	TPFVAPTNDVILR
	SDMTVEER	
	AELGQMDSAR	
	ARSDMTVEER	
	AKAELGQMDSAR	
	NSQHKFEDTWHEQQPIELVLGK	
Cover (%)	13.4	3

B



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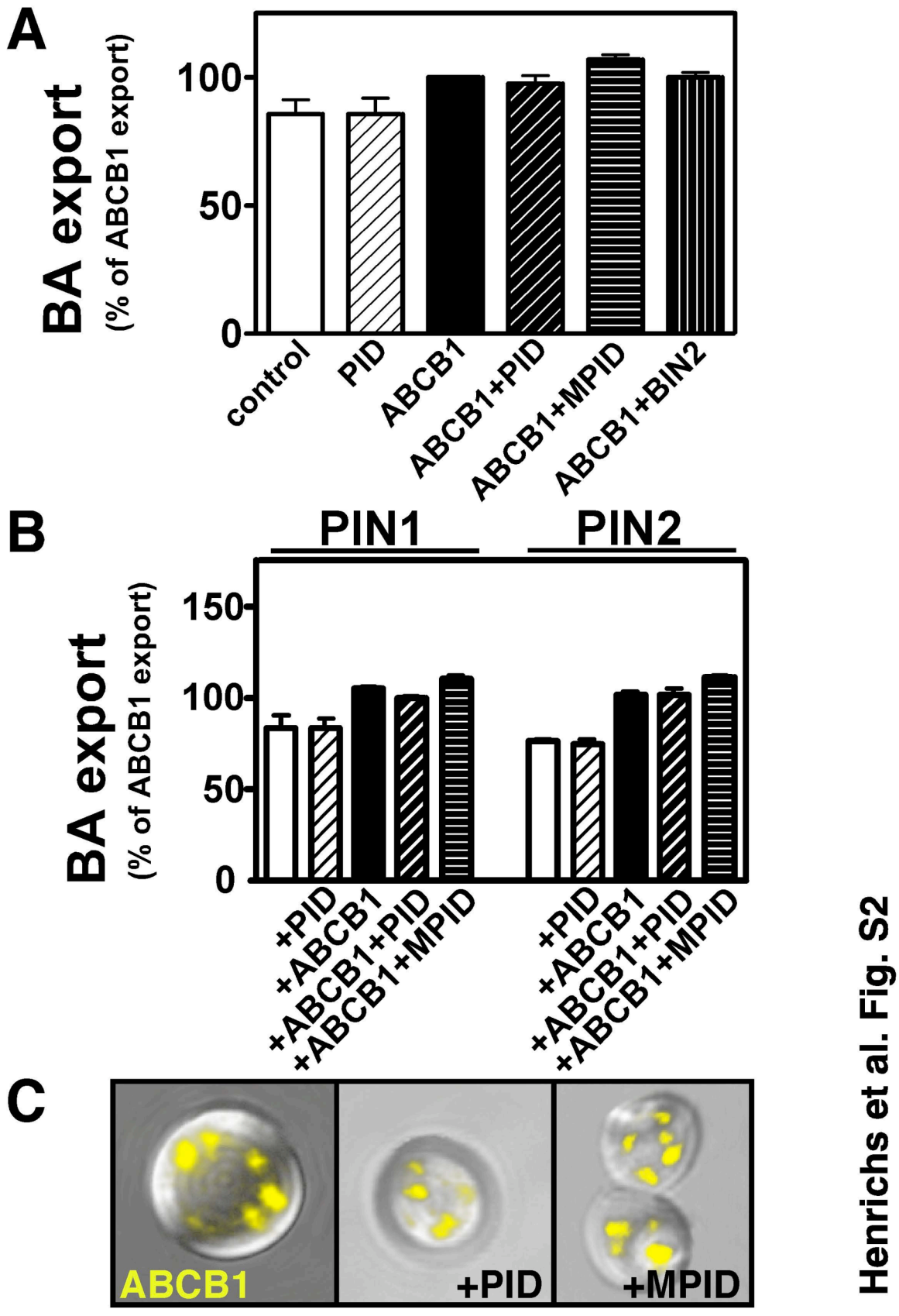

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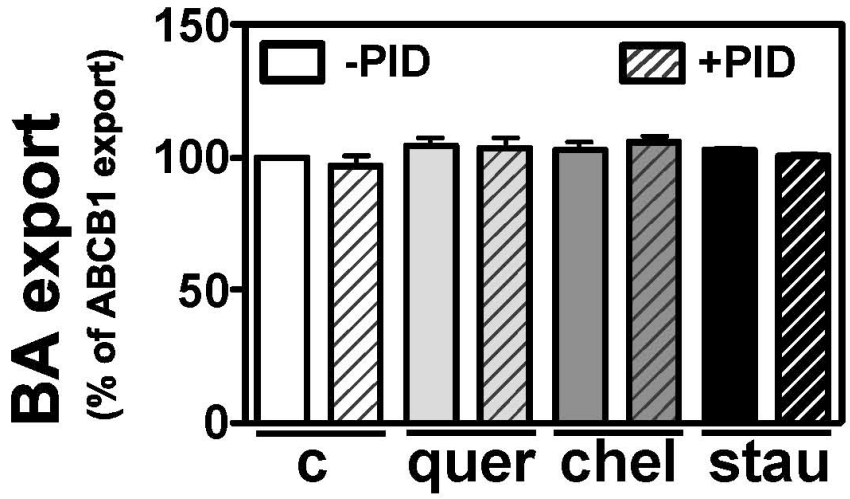

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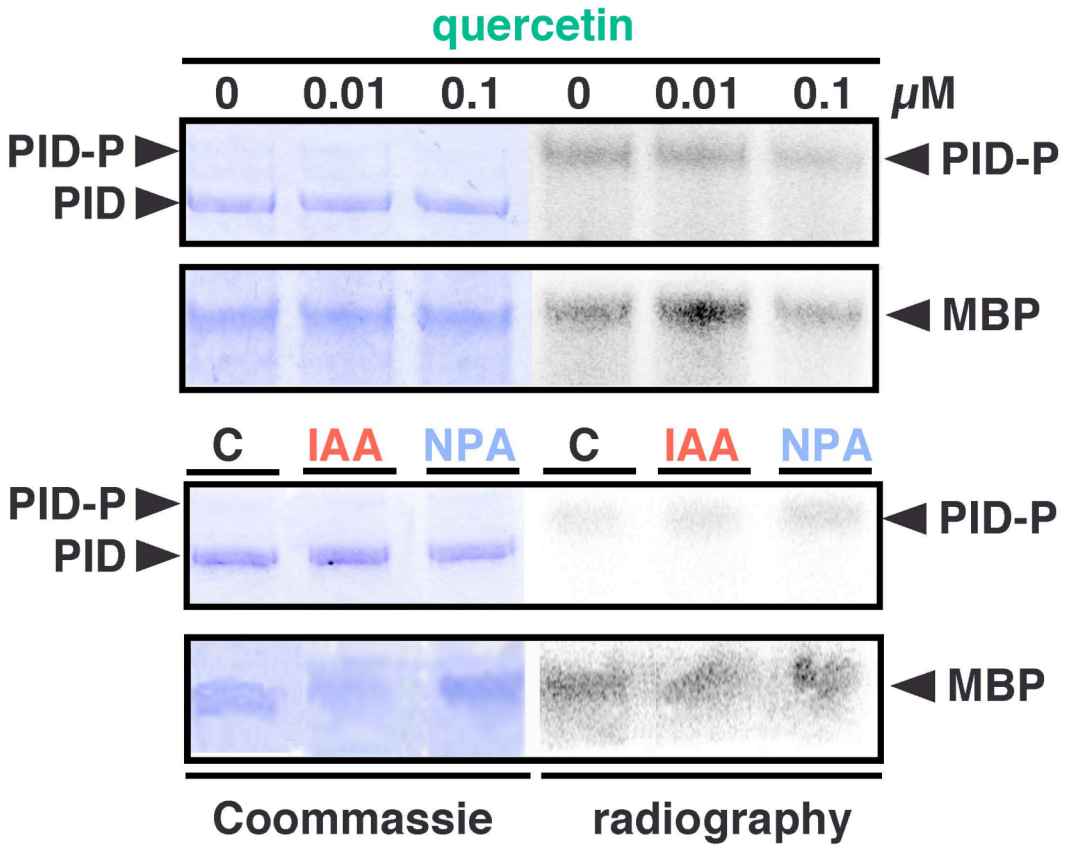

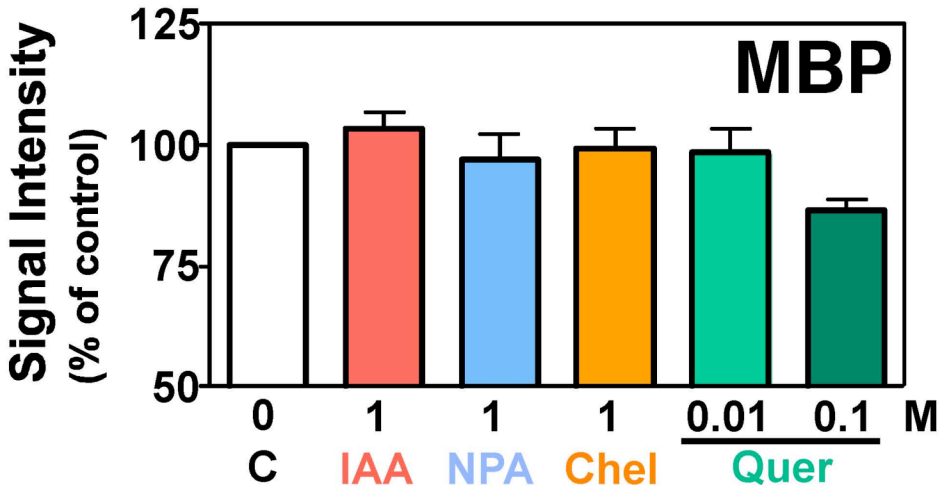

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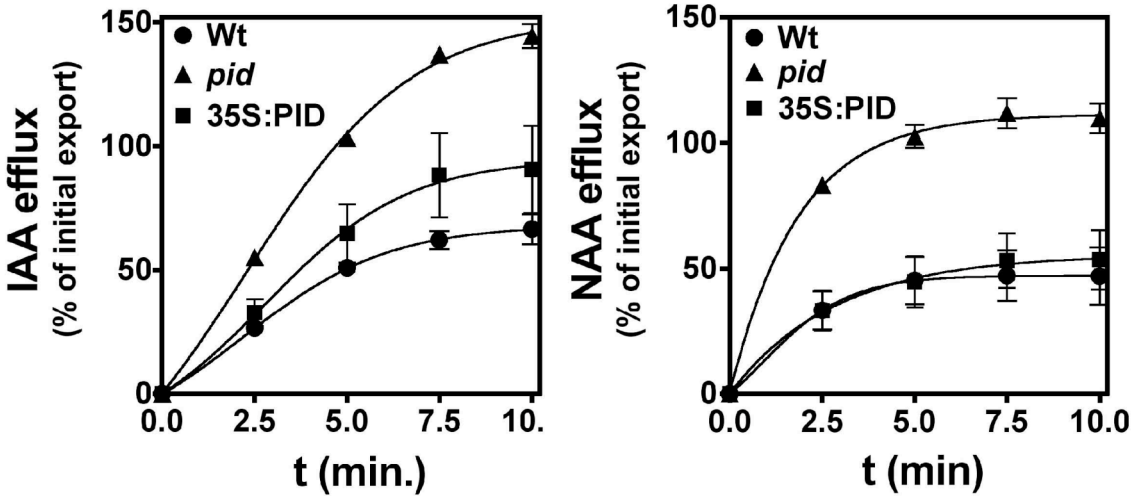

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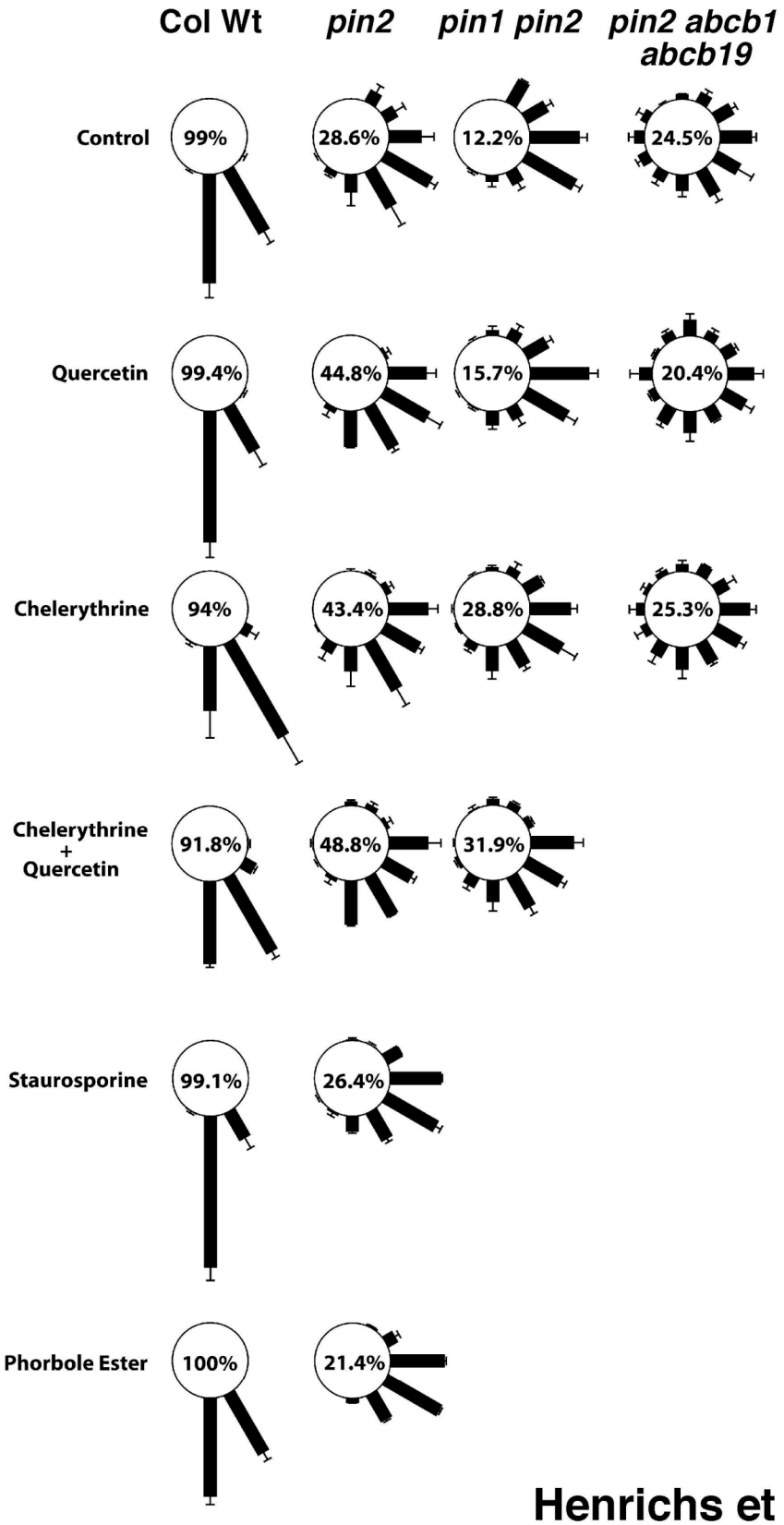
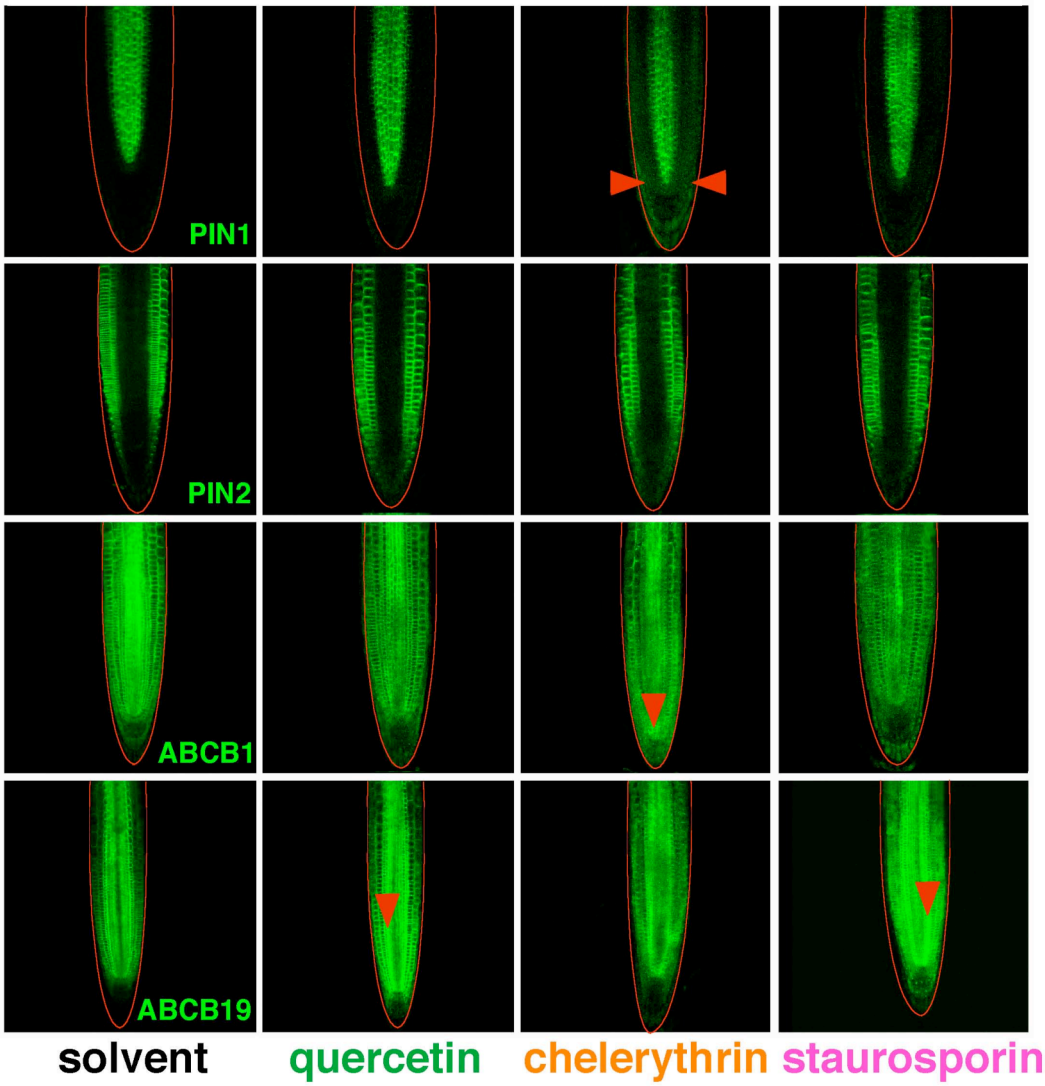


Henrichs et al. Fig. S6



Henrichs et al. Fig. S7

6 DISCUSSION

The maintainance of intercellular auxin gradients by auxin transport is regulated basically on each molecular level - from transcriptional gene regulation to a variety of post-transcriptional modifications. This ability demonstrates the impressive competence plants have evolved in order to maintain developmental and physiological plasticity.

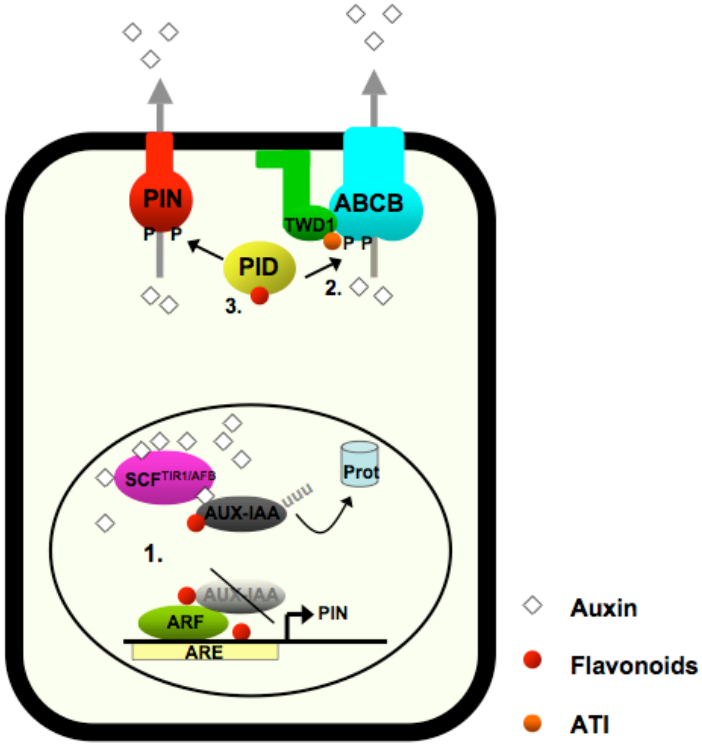


Figure 14. Flavonoids fine-tune auxin responses on different molecular levels

1. Flavonoids regulate transcription of auxin-relevant genes. Whether this involves a direct interaction with specific promoter elements or indirect effects involving components of the signaling pathways is still unknown. It is likely that flavonoids affect gene transcription on different levels: by direct binding to the Auxin Responsive Element (ARE) or probably by indirect modifications via binding to the Aux/IAA transcriptional repressors or the Auxin Response Factor (ARF) regulators. Alternatively, flavonoids may also use a completely different and so far unknown signaling pathway.
2. Auxin transport inhibitors (ATIs), like BUM, modulate the ABCB1-mediated auxin transport activity and compete with NPA for the same binding site. Furthermore NPA and BUM have a destabilizing effect on the TWD1-ABCB1 protein-protein interaction.
3. Flavonoids are well known as kinase inhibitors and bind directly to PID as negative regulators.

P: phosphate, Prot: proteasome, U: ubiquitin.

In this context the three different projects of my PhD studies reflect the huge variation of possible regulatory mechanisms that affect the auxin transport machinery. As plant endogenous compounds, flavonoids represent a huge variety of different modulatory abilities to fine-tune the auxin distribution at various molecular levels, from transcriptional gene regulation to auxin transport- or as kinase inhibitors (Figure 14). The regulatory impact of these on auxin transport regulation was analyzed from different perspectives. The first project demonstrates that flavonoids regulate, by a yet unknown mechanism, gene expression of a PIN protein that finally leads to changes in plant growth responses. In my second project, the detailed analysis of the newly identified auxin transport inhibitor, BUM, on plant physiology and further on molecular targets demonstrates that BUM affects the auxin transport machinery directly by interaction with the auxin transporter ABCB1. Here, modification of transporter activity leads to changes in auxin movements and, as a consequence, to different plant responses. Finally, in the third project, the regulation of protein transport activity by protein phosphorylation events

was analyzed. Reversible protein phosphorylation is known to induce rapid changes to proteins that open the possibility to react fast and flexibly to changing conditions, compared to protein trafficking for example. During the regulation of auxin transport, the kinase PINOID controls auxin transport activity of different efflux proteins via protein phosphorylation. Upstream of the regulatory pathway flavonoids negatively regulate PID, most probably by direct drug binding.

However, taking all this into account, research on auxin regulatory mechanism helps to understand general cellular responses and mechanisms.

6.1 Flavonoids modulate Gene Expression of PIN1

The first project of my PhD studies demonstrates that defects in the gravitropic response in the *pin2* mutant background are linked to an altered flavonoid accumulation in the elongation zone of the root tip (Santelia et al., 2008). Flavonoid stainings (DPBA) showed an increase of flavonoid accumulation in the elongation zone of gravistimulated wild type roots, an effect that was only weakly detected in *pin2.* These data already indicate a regulatory function for flavonoids during gravitropic root bending. However, the agravitropic phenotype of *pin2* results mainly from a symmetric distribution of auxin in the root tip that prevents cell elongation in the upper cell of the lateral root cap and therefore bending of the root tip along the gravitropic vector. Interestingly, application of a low concentration of quercetin partially recovers the gravitropic response in *pin2* by restoration of the asymmetric basipetal auxin transport in the lower cells of the lateral root cap. Here, flavonoids do not act as PAT inhibitors because they simply had no effect on the gravitropic response of wild type plants and NPA failed to restore the *pin2* phenotype.

Detailed analysis showed that low concentrations of flavonoids induce the ectopic expression of PIN1 to replace PIN2 in the appropriate tissue in order to rescue the gravitropic response (Santelia et al., 2008). Quantification of confocal microscopy analysis showed that the polar localization of PIN1 correlates with the asymmetric auxin gradient. This finding indicates that flavonoids do not solely inhibit efflux transporters, but also resemble a function as modulators of gene expression and cellular protein trafficking. Moreover, these findings support the suggestion that flavonoids might have an opposite effect on PIN regulation: a positive effect on PIN1 and a negative effect on PIN2. These results go in hand with previously published data, where opposite regulation of PIN1 and PIN2 by flavonoids was reported: PIN1 is delocalized from the plasma membrane and gene expression is depressed in the absence of flavonoids, whereas localization of PIN2 is unaffected and gene expression is enhanced (Peer et al., 2004; Peer et al., 2001; Peer and Murphy, 2007). Further analysis suggested that changes in PIN gene expression are possibly linked to an altered auxin transport rather than to a flavonoid effect because changes were mimicked by exogenous application of auxin. However, the reported changes in PIN gene expression were only detected in roots, and not in shoots, where, under wild-type conditions, flavonoid staining shows an intense accumulation specifically in the root elongation zone (Peer et al., 2001).

In our scenario, exogenously applied quercetin induces the ecotopic expression of PIN1 by an unknown mechanism. Interestingly, recent reports from a number of different plant species have documented the presence of flavonoids in nuclei, raising the possibility of a function as transcriptional mediators (Feucht et al., 2004). Although the physiological roles of flavonoids in plant cell nuclei are far from clear, it has been suggested that these compounds may serve to protect DNA from UV and oxidative damage (Feucht and Polster, 2001) or, directly or indirectly, control the transcription of genes required for growth and development including those encoding auxin transport proteins (Buer and Muday, 2004; Saslowsky et al., 2005). There is also mounting evidence that flavonoids can interact with specific proteins in animal cells, including nuclear components such as histones (Ramadass et

al., 2003) and a subset of mammalian DNA polymerases (Mizushina et al., 2003). Further, quercetin has been reported to bind specific target proteins in *Drosophila* nuclei (Gutzeit et al., 2004) and in addition it has been shown to bind directly to DNA (Walle et al., 2003). There are also numerous reports of the activation of gene expression in plant and animal cells by flavonoids (Wietzke and Welsh, 2003), although whether this involves a direct interaction with specific promoter elements or indirect effects involving components of signaling pathways is still unknown. Interestingly, quercetin inhibits the expression of specific oncogenes and genes controlling the cell cycle and also reciprocally up-regulates the expression of several tumor suppressor genes (Woo et al., 2005). Examples from different organisms and systems indicate that flavonoids can be generally considered as transcriptional regulators. In the case of auxin transcriptional regulation it is likely that flavonoids affect gene transcription on different levels: by direct binding to the Auxin Responsive Element (ARE) or, probably by indirect modifications, via binding to the Aux/IAA transcriptional repressors or the ARF regulators. Alternatively, flavonoids may also use a completely different and so far unknown signaling pathway (Figure 14).

6.2 Identification of an ABCB-specific auxin transport inhibitor

In my second project chemical libraries of small organic compounds were screened for novel plant physiological and developmental regulators. We identified a novel, highly potent ATI, called BUM (Figure 11). We analyzed the effect of BUM on auxin-regulated plant physiology and development in detail and compared the various effects with NPA, a well-characterized ATI (Bailly et al., 2008).

BUM is a highly potent auxin transport with an IC_{50} that is approximately 30 times lower than the one of NPA. Although BUM and NPA cause quiete similar phenotypes in wild-type plants, they also induce complementary effects. Application of BUM and NPA to wild-type plants induces a pin-formed shoot phenotype, but BUM additionally causes strongly reduced root growth, which is different to NPA. Moreover BUM lacks growth activation at lower concentrations while NPA shows a stimulating and inhibitory effect on hypocotyl elongation under light conditions at different concentrations. Also shoot hook formation and opening of etiolated seedlings were inhibited by BUM, but not by NPA. In general, root and hypocotyl growth inhibition by BUM and NPA was light dependent, which is in agreement with the concept that in light-grown seedlings, elongation and bending responses are primarily controlled by PAT.

Interestingly, the loss-of *ABCB1* mutation confers insensitivity towards BUM during lateral root emergence, but not during root gravitropism. It is known that lateral root emergence requires shoot-derived auxin that is transported by ABCB1 and as a consequence *abcb1* mutants show a reduced number of lateral roots. Taking this into account, it is likely that BUM specifically inhibits the ABCB1-mediated auxin transport during lateral root development.

Physiological and auxin transport analysis, as well as binding assays, identified ABCB1, and to a lesser extend ABCB19, as key targets of BUM, without affecting other components of the auxin transport machinery, nor influencing ABCB protein expression or abundance, as in the case of Gravacin (Rojas-Pierce et al., 2007). Interestingly, BUM, like NPA, interferes with PIN expression, an effect that might be indirectly triggered by elevated auxin levels. Furthermore, BUM and NPA have a destabilizing effect on the ABCB1-TWD1 protein-protein interaction. Based on docking studies on the putative *Arabidopsis* ABCB1 structure, modeled on the crystal structure of ABCB-related multidrug efflux pump Sav1866, BUM probably binds, like NPA, to the second NBD of ABCB1. Interestingly, BUM additionally has a high affinity (around -50 kcal/mol) to a second NBD1-NBD2 interface where no NPA binding was predicted; this might account for its severe inhibition.

As a consequence, with BUM we now have nice tool in our hands that helps to dissect auxin transport routes, which in this case affect shoot-derived auxin movements that induce lateral root development. Furthermore, BUM specifically inhibits ABCB1 without affecting PIN proteins, which makes it attractive for further studies on ABCB1 activities. Moreover, BUM is complementary to NPA and the recently identified ATI, gravacin, by showing distinct ABCB target spectra and by lack of interference with ABCB membrane trafficking.

Finally it is important to point out that both BUM and NPA mimic, a mutant phenotype in wild-type plants, here *pin1*, that does not necessarily represent the corresponding binding protein for these ATIs. In this case phenotypic observation did not neccessarily implicate the binding target and therefore opens an important discussion about the overall regulation of auxin transport by protein complexes. Several lines of evidence suggest that PIN proteins do not themselves act as NPA binding proteins (Lomax et al., 1995, Kim and Henrichs et al., 2009 (under revision) nor does NPA directly affect PIN protein cycling (Petrasek et al., 2003). Instead, ABCB1 and ABCB19 have been identified as high-affinity NPA binding proteins (Rojas-Pierce et al., 2007) and as targets of NPA (Murphy et al., 2002; Noh et al., 2001). Additionally NPA also binds TWD1 and application of NPA leads to a destabilizing effect on the physical ABCB1-TWD1 interaction (Bailly et al., 2008), suggesting that TWD1 and ABCB1 represent high- and low-affinity components of the NPA-sensitive efflux machinery (Figure 10). Taking this into account our data further support a scenario where the pin-formed phenotype, which can be induced by NPA and BUM, results at first hand from the inhibition of an interacting protein of PIN1, most probably ABCB1 or ABCB19 and/or TWD1.

In general it is not surprising that ATIs may have many different modes of action. Taking NPA, TIBA, BUM and Gravacin as an example shows that a more detailed analysis of ATIs helps to understand unknown mechanisms of the auxin transport complex and its interacting proteins. As a consequence, forward chemical genetics, utilizing small molecules to perturb a pathway to allow the identification of important genes during development, is a useful instrument in order to screen for new potent inhibitors of the auxin transport machinery. Chemical genomics is also a powerful tool by means of avoiding problems that are faced by classical genomics such as gene lethality and redundancy (Surpin et al., 2005). Furthermore ATIs are effective not only in plant cells but also in yeast and mammals. This evolutionarily conserved action might therefore be useful in order to extend our knowledge about cellular mechanisms in general (Dhonukshe et al., 2008).

6.3 Regulation of auxin transport activity by protein phosphorylation

Several studies indicate that reversible protein phosphorylation is an important regulatory mechanism for auxin transport. Detailed analysis revealed that direct phosphorylation by the PINOID kinase is an important signal in PIN polar targeting (Figure 12) (Michniewicz et al., 2007). However, it is an open question if phosphorylation of PIN proteins is sufficient to change auxin transport routes, or if more components and other possible phosphorylation targets are involved.

Several lines of clinical evidence suggest ABCBs as general targets for phosphorylation dependent regulation in a so-called linker region that modifies their transport and associated ATPase activity (Idriss et al., 2000; Szabo et al., 1997). An accumulation of phosphorylatble serine residues was identified to be phosphorylated by protein kinase A and/or C (PKA/C), orthologues of the plant kinase PINOID (Chambers et al., 1990; Conseil et al., 2001; Orr et al., 1993), that regulate the drug transport properties (Szabo et al., 1997). During my third project, we addressed the key question if PID regulates transporter activities as well as PIN protein locations.

As a starting point of this work we identified the PID kinase as an TWD1 interacting protein in a tandem affinity purification approach, where we used TAP-tagged TWD1 as a bait. These data could be further underlined by protein interaction analysis (BRET) where an interaction of TWD1 and PID was detected *in planta*. Due to the fact that TWD1 functionally interacts with ABCB1, we considered also an effect of PID on ABCB1 transport activity. Co-expression of ABCB1 and PID in yeast leads to inhibition of the ABCB1-mediated auxin efflux. Interestingly, the inhibitory effect of PID on PIN proteins was only detected when they were expressed in combination with ABCB1. These effects on transport activities are specific and clearly linked to PID-mediated phosphorylation events, because co-expression of ABCB1/PIN1 with an inactive form of PID (MPID) showed no effects. BIN2, a kinase known to regulate brassinosteroid signaling, had no effect on the transport activities. When we repeated the experiments in tobacco protoplasts, PID counter-actively regulated ABCB1 and PIN1: PID drastically stimulates ABCB1- and inhibits PIN1-mediated export. This was surprising, because PID is generally accepted as a positive regulator for auxin transport. In agreement to our findings, protoplasts of *pid* Arabidopsis mutants showed an drastically elevated auxin efflux activity compared to wild type, whereas over-expression of PID had no significant effect. However, transport assays in yeast and tobacco cells indicate that co-expression of PID with ABCB1 or PIN1 leads to drastic changes in the auxin transport activities. Surprisingly, PID had an inhibitory effect on ABCB1 in the yeast system, whereas activation was measured in tobacco protoplasts. These conflicting results might indicate a missing regulatory parameter and, considering the TWD1 and PID protein interaction data, a promising candidate in this case might be TWD1 itself. Taking into account that ABCB1 and TWD1 directly interact, our data suggest that TWD1 acts might act as a “bridging protein” between PID and ABCB1. The impact of TWD1 on PID recruitment during auxin ABCB1 regulation is still an open question and is currently being addressed.

Interestingly, we identified quercetin as an upstream regulator of PID that negatively affects its activity, probably by direct binding. Application of quercetin blocks the inhibitory effect of PID on the ABCB1-

mediated transport activity and as a consequence results in a fully active ABCB1. *In vitro* phosphorylation analysis revealed that quercetin inhibits PID autophosphorylation in a concentration-dependent manner and binding studies showed a direct binding of quercetin to PID. Although IAA showed a weak binding affinity to PID, other tested compounds, like NPA or NAA, showed no binding. In line with these data, microsomes from *pid* seedlings showed almost no quercetin binding, whereas over-expression of PID leads to an enhanced quercetin binding capacity. Finally, our data suggest a model where quercetin directly binds PID in order to act as an endogenous kinase inhibitor to fine-tune the auxin transport machinery by means of reversible protein phosphorylation events that allow reacting according to rapidly changing conditions.

Surprisingly binding studies with NPA showed exactly the opposite effect compared to quercetin. Although these results are far from clear, it is possible that PID directly or indirectly affects the NPA binding capacity by modulating the abundance of NPA binding proteins or by induction of conformational changes in their protein structure. However, these data again indicate the regulation of auxin transport by a functional efflux protein complex that is composed of PINs, ABCBs- and TWD1 and additional regulatory proteins.

6.4 Conclusion and Outlook

Calcium is the Key!

Upstream events regulating PID activity reveal interesting links to possible unknown regulatory modifications of the auxin transport machinery. It seems that auxin induces the regulation of PID by accumulation of calcium in the cell (Toyota et al., 2008). PID is negatively regulated by TCH3 or positively by PBP1 (Figure 12). Interestingly, TCH3 is a calmodulin-related protein and as a consequence calmodulin inhibitors enhance PID activity (Sistrunk et al., 1994). It might be worth considering that TWD1 is possibly a potential binding protein for TCH3, as calmodulin-binding to its CaM-binding domain in its C-terminus has been (Figure 15) (Geisler et al., 2003; Kamphausen et al., 2002). Studies on the human homologue of TWD1, FKBP38, show that function of HsFKBP38 requires a prior activation by the calcium-sensor calmodulin. As a consequence the peptidyl prolyl *cis/trans*-isomerase (PPIase) activity of HsFKBP38 is induced by the FKBP38/Calmodulin/Calcium complex in order to affect conformational changes (Edlich et al., 2007). Taking the human FKBP38 as an example it becomes likely that conformational changes of TWD1 are induced by calcium-dependent binding of a calmodulin-like protein, such as TCH3, that affects PID activity and thus auxin transport. Concerning our results from the TAP-TWD1 approach one might consider that TWD1 might indirectly bind PID by interaction with TCH3 (Figure 15). Therefore, it is of major interest to investigate a possible interaction of TWD1 and TCH3 and to further analyze its regulatory impact on PID.

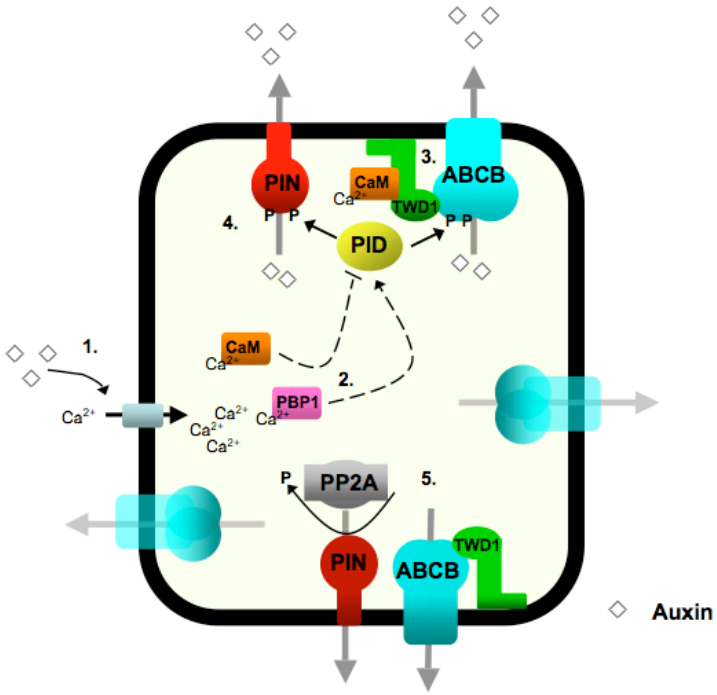


Figure 15. Outlook of the auxin transport protein complex and its regulation by modulatory elements

1. Elevated auxin levels increase the cytosolic calcium concentration
2. PID activity is calcium-dependently regulated, positively or negatively by the calcium-binding proteins PINOID-BINDING PROTEIN 1 (PBP1) and a calmodulin-like protein (CaM)., like TOUCH3 (TCH3).
3. PID probably regulates the activity of the ABCB protein complex by phosphorylation of ABCB1 in the linker domain. CaM interacts with the calmodulin-binding domain of TWD1 that further interacts with the C-terminal domain of ABCB1. TWD1 has a positive regulatory effect on ABCB1-mediated auxin transport; the effect of CaM on TWD1 is still unclear.
4. PID regulates the protein localization of PIN proteins by reversible phosphorylation.
5. Dephosphorylation of PIN proteins by the phosphatase PP2A leads to a top-to-bottom switch of PIN proteins

6.5 WAG kinases regulate an NPA-binding protein

PID groups to the AGC3 clade together with AGC3-4, WAG1 and WAG2. Although several lines of evidence indicate also a function for WAG kinases during auxin transport, the regulatory impact of WAGs on the auxin transport machinery remains less clear. Loss-of-function mutations of *wag1 wag2* show an auxin-dependent root waving phenotype (Santner and Watson, 2006) and root curling is more resistant to NPA. Enhanced NPA sensitivity, the fact that the WAG kinases, like PID, are plasma membrane-associated and the enhanced expression in the root tips suggest that PID and WAG kinases act in the same or in a parallel pathway to regulate the auxin transport machinery, probably by the regulation of NPA-binding proteins, like ABCB1 or interactors, like TWD1. Taking into account that members of the AGC3 clade show a certain redundancy during development and the calcium-dependent regulation by TCH3 and PBP1 is conserved for all four AGC3 kinases (Offringa, unpublished), a possible function of WAG kinases in auxin transport regulation has to be considered. Testing the activity of different PINs and ABCBs in combination with AGC3 kinases will give new insightes into possible regulatory pathways. Also the effect of WAG kinases on PIN protein polarity is a question that needs to be addressed. Furthermore, as WAG kinases are rapidly down-regulated by light and show more pronounced on root growth, they probably play a role during gravitropism or root development, responses where PID plays a limited role.

In summary, phosphorylation influences the polar localization of PIN proteins and additionally affects transport activity of efflux proteins and therefore the redistribution of auxin. According to the fact that protein phosphorylation leads to rapid changes in protein activity, this mode of action offers an economical way to quickly respond to environmental changes. Also here the different combination of PIN and ABCB protein may lead to flexible responses that might be regulated by different members of the AGC3 kinases.

Feedback on Auxin

All research tools suffer from the limitation of possible components that can be analyzed at a time. In case of auxin research this is definitely a drawback, concerning the amount of possible regulatory pathway it controls (Figure 13). During the last decade, an incredible amount of different projects tried to shed light on the function of auxin by addressing many different aspects. Finally, as a general conclusion it seems that at the end auxin controls its own biosynthesis, homeostasis and transport by several and also interconnected regulatory feedback loops. In terms of regulation, auxin is able to compose the set of its own regulatory machinery according to the developmental stage of the cell and the environmental conditions of the plant. As a consequence, the individual proteins interact, interfere and regulate each other in order to mediate fine-tuning of the auxin distribution pattern.

Furthermore, endogenous compounds, like flavonoids, are also part of this regulatory network of the auxin transport machinery (Figure 14). The modulatory effect of flavonoids on auxin transport might result from a combinatory effect on ABCB activity and its interaction with TWD1 and additionally on PIN gene expression and cellular trafficking. Our findings that flavonoids have a regulatory role on

phosphorylation dependent events regulating auxin transport by a direct interaction with PID are therefore inline with their well-known function as kinase inhibitors (Gamet-Payrastre et al., 1999).

Finally, it is important to point out that understanding plant ABCB transporter function and regulation in more detail provides multiple benefits at one time. ABCBs are not only of agricultural interests but also of clinical relevance as human ABCBs are the primary cause of multidrug resistance (MDR) phenomena during chemotherapeutic treatment of cancer. Unlike in plants, mammalian ABCBs seem to exhibit a more unspecific substrate specificity The best-described member of the ABCB subgroup is the human ABCB1 protein, which has been extensively studied for its role in increased MDR resulting from its overexpression in cancer cells. Moreover, P-glycoproteins are well-known detoxifiers by exporting different chemically unrelated toxins (Ambudkar et al., 2003). Further, human ABCs are also involved in inherited diseases like cystic fibrosis or tangier disease (Borst et al., 2000). Modification of the transport capacity by identifying and understanding the mechanisms of regulatory components (like intereacting kinases) or drug-like modulators (like quercetin), might lead help developing novel stategies to overcome MDR, for example by application of inhibitory drugs or post-translational modifications.

7 REFERENCES

Abas, L., Benjamins, R., Malenica, N., Paciorek, T., Wisniewska, J., Moulinier-Anzola, J.C., Sieberer, T., Friml, J., and Luschnig, C. (2006). Intracellular trafficking and proteolysis of the Arabidopsis auxin-efflux facilitator PIN2 are involved in root gravitropism. Nat Cell Biol *8*, 249-256.

Agullo, G., Gamet-Payrastre, L., Manenti, S., Viala, C., Remesy, C., Chap, H., and Payrastre, B. (1997). Relationship between flavonoid structure and inhibition of phosphatidylinositol 3-kinase: a comparison with tyrosine kinase and protein kinase C inhibition. Biochem Pharmacol *53*, 1649-1657.

Ahmad, S., Safa, A.R., and Glazer, R.I. (1994). Modulation of P-glycoprotein by protein kinase C alpha in a baculovirus expression system. Biochemistry *33*, 10313-10318.

Aloni, R., Aloni, E., Langhans, M., and Ullrich, C.I. (2006). Role of auxin in regulating Arabidopsis flower development. Planta *223*, 315-328.

Ambudkar, S.V., Kimchi-Sarfaty, C., Sauna, Z.E., and Gottesman, M.M. (2003). P-glycoprotein: from genomics to mechanism. Oncogene *22*, 7468-7485.

Bailly, A., Sovero, V., Vincenzetti, V., Santelia, D., Bartnik, D., Koenig, B.W., Mancuso, S., Martinoia, E., and Geisler, M. (2008). Modulation of P-glycoproteins by auxin transport inhibitors is mediated by interaction with immunophilins. J Biol Chem *283*, 21817-21826.

Bainbridge, K., Guyomarc'h, S., Bayer, E., Swarup, R., Bennett, M., Mandel, T., and Kuhlemeier, C. (2008). Auxin influx carriers stabilize phyllotactic patterning. Genes Dev *22*, 810-823.

Baluska, F., Barlow, P.W., Baskin, T.I., Chen, R., Feldman, L., Forde, B.G., Geisler, M., Jernstedt, J., Menzel, D., Muday, G.K.*, et al.* (2005). What is apical and what is basal in plant root development? Trends Plant Sci *10*, 409-411.

Bandyopadhyay, A., Blakeslee, J.J., Lee, O.R., Mravec, J., Sauer, M., Titapiwatanakun, B., Makam, S.N., Bouchard, R., Geisler, M., Martinoia, E.*, et al.* (2007). Interactions of PIN and PGP auxin transport mechanisms. Biochem Soc Trans *35*, 137-141.

Baudouin, E., Charpenteau, M., Ranjeva, R., and Ranty, B. (2002). A 45-kDa protein kinase related to mitogen-activated protein kinase is activated in tobacco cells treated with a phorbol ester. Planta *214*, 400-405.

Benjamins, R., Ampudia, C.S., Hooykaas, P.J., and Offringa, R. (2003). PINOID-mediated signaling involves calcium-binding proteins. Plant Physiol *132*, 1623-1630.

Benjamins, R., Quint, A., Weijers, D., Hooykaas, P., and Offringa, R. (2001). The PINOID protein kinase regulates organ development in Arabidopsis by enhancing polar auxin transport. Development *128*, 4057-4067.

Benjamins, R., and Scheres, B. (2008). Auxin: the looping star in plant development. Annu Rev Plant Biol *59*, 443-465.

Benkova, E., Michniewicz, M., Sauer, M., Teichmann, T., Seifertova, D., Jurgens, G., and Friml, J. (2003). Local, efflux-dependent auxin gradients as a common module for plant organ formation. Cell *115*, 591-602.

Bennett, M.J., Marchant, A., Green, H.G., May, S.T., Ward, S.P., Millner, P.A., Walker, A.R., Schulz, B., and Feldmann, K.A. (1996a). Arabidopsis AUX1 gene: a permease-like regulator of root gravitropism. Science *273*, 948-950.

Bennett, M.J., Marchant, A., Green, H.G., May, S.T., Ward, S.P., Millner, P.A., Walker, A.R., Schulz, B., and Feldmann, K.A. (1996b). Arabidopsis AUX1 gene: A permease-like regulator of root gravitropism. Science *273*, 948-950.

Bennett SRM, Alvarez J, Bossinger G, Smyth DR (1995) Morphogenesis in *pinoid* mutants of *Arabidopsis thaliana*. Plant J. 8: 505-520

Berger, S. (2002). Jasmonate-related mutants of Arabidopsis as tools for studying stress signaling. Planta *214*, 497-504.

Bernasconi, P., Patel, B.C., Reagan, J.D., and Subramanian, M.V. (1996). The N-1-Naphthylphthalamic Acid-Binding Protein Is an Integral Membrane Protein. Plant Physiol *111*, 427-432.

Birnbaum, K., Shasha, D.E., Wang, J.Y., Jung, J.W., Lambert, G.M., Galbraith, D.W., and Benfey, P.N. (2003). A gene expression map of the Arabidopsis root. Science *302*, 1956-1960.

Blakeslee, J.J., Bandyopadhyay, A., Lee, O.R., Mravec, J., Titapiwatanakun, B., Sauer, M., Makam, S.N., Cheng, Y., Bouchard, R., Adamec, J*., et al.* (2007). Interactions among PIN-FORMED and P-glycoprotein auxin transporters in Arabidopsis. Plant Cell *19*, 131-147.

Blilou, I., Xu, J., Wildwater, M., Willemsen, V., Paponov, I., Friml, J., Heidstra, R., Aida, M., Palme, K., and Scheres, B. (2005). The PIN auxin efflux facilitator network controls growth and patterning in Arabidopsis roots. Nature *433*, 39-44.

Blobe, G.C., Sachs, C.W., Khan, W.A., Fabbro, D., Stabel, S., Wetsel, W.C., Obeid, L.M., Fine, R.L., and Hannun, Y.A. (1993). Selective regulation of expression of protein kinase C (PKC) isoenzymes in

multidrug-resistant MCF-7 cells. Functional significance of enhanced expression of PKC alpha. J Biol Chem *268*, 658-664.

Bogre, L., Okresz, L., Henriques, R., and Anthony, R.G. (2003). Growth signalling pathways in Arabidopsis and the AGC protein kinases. Trends Plant Sci *8*, 424-431.

Borst, P., Evers, R., Kool, M., and Wijnholds, J. (2000). A family of drug transporters: the multidrug resistance-associated proteins. J Natl Cancer Inst *92*, 1295-1302.

Bouchard, R., Bailly, A., Blakeslee, J.J., Oehring, S.C., Vincenzetti, V., Lee, O.R., Paponov, I., Palme, K., Mancuso, S., Murphy, A.S.*, et al.* (2006). Immunophilin-like TWISTED DWARF1 modulates auxin efflux activities of Arabidopsis P-glycoproteins. J Biol Chem *281*, 30603-30612.

Brown, D.E., Rashotte, A.M., Murphy, A.S., Normanly, J., Tague, B.W., Peer, W.A., Taiz, L., and Muday, G.K. (2001). Flavonoids act as negative regulators of auxin transport in vivo in arabidopsis. Plant Physiol *126*, 524-535.

Buer, C.S., and Muday, G.K. (2004). The transparent testa4 mutation prevents flavonoid synthesis and alters auxin transport and the response of Arabidopsis roots to gravity and light. Plant Cell *16*, 1191-1205.

Buer, C.S., Muday, G.K., and Djordjevic, M.A. (2007). Flavonoids are differentially taken up and transported long distances in Arabidopsis. Plant Physiol *145*, 478-490.

Castro, A.F., Horton, J.K., Vanoye, C.G., and Altenberg, G.A. (1999). Mechanism of inhibition of P-glycoprotein-mediated drug transport by protein kinase C blockers. Biochem Pharmacol *58*, 1723-1733.

Chambers, T.C., McAvoy, E.M., Jacobs, J.W., and Eilon, G. (1990). Protein kinase C phosphorylates P-glycoprotein in multidrug resistant human KB carcinoma cells. J Biol Chem *265*, 7679-7686.

Chambers, T.C., Pohl, J., Raynor, R.L., and Kuo, J.F. (1993). Identification of specific sites in human P-glycoprotein phosphorylated by protein kinase C. J Biol Chem *268*, 4592-4595.

Chambers, T.C., Zheng, B., and Kuo, J.F. (1992). Regulation by phorbol ester and protein kinase C inhibitors, and by a protein phosphatase inhibitor (okadaic acid), of P-glycoprotein phosphorylation and relationship to drug accumulation in multidrug-resistant human KB cells. Mol Pharmacol *41*, 1008-1015.

Chandler, J.W. (2009). Local auxin production: a small contribution to a big field. Bioessays *31*, 60-70.

Chaudhary, P.M., and Roninson, I.B. (1992). Activation of MDR1 (P-glycoprotein) gene expression in human cells by protein kinase C agonists. Oncol Res *4*, 281-290.

Cheng, S.H., Rich, D.P., Marshall, J., Gregory, R.J., Welsh, M.J., and Smith, A.E. (1991). Phosphorylation of the R domain by cAMP-dependent protein kinase regulates the CFTR chloride channel. Cell *66*, 1027-1036.

Cheng, Y., Dai, X., and Zhao, Y. (2006). Auxin biosynthesis by the YUCCA flavin monooxygenases controls the formation of floral organs and vascular tissues in Arabidopsis. Genes Dev *20*, 1790-1799.

Cheng, Y., Dai, X., and Zhao, Y. (2007a). Auxin synthesized by the YUCCA flavin monooxygenases is essential for embryogenesis and leaf formation in Arabidopsis. Plant Cell *19*, 2430-2439.

Cheng, Y., Qin, G., Dai, X., and Zhao, Y. (2007b). NPY1, a BTB-NPH3-like protein, plays a critical role in auxin-regulated organogenesis in Arabidopsis. Proc Natl Acad Sci U S A *104*, 18825-18829.

Christensen, S.K., Dagenais, N., Chory, J., and Weigel, D. (2000). Regulation of auxin response by the protein kinase PINOID. Cell *100*, 469-478.

Conseil, G., Perez-Victoria, J.M., Jault, J.M., Gamarro, F., Goffeau, A., Hofmann, J., and Di Pietro, A. (2001). Protein kinase C effectors bind to multidrug ABC transporters and inhibit their activity. Biochemistry *40*, 2564-2571.

Darwin, C., and Darwin, F. (1880). The Power of Movements in Plants. Murray, London.

Delbarre, A., Muller, P., and Guern, J. (1998). Short-Lived and Phosphorylated Proteins Contribute to Carrier-Mediated Efflux, but Not to Influx, of Auxin in Suspension-Cultured Tobacco Cells. Plant Physiol *116*, 833-844.

Delker, C., Raschke, A., and Quint, M. (2008). Auxin dynamics: the dazzling complexity of a small molecule's message. Planta *227*, 929-941.

Dello Ioio, R., Nakamura, K., Moubayidin, L., Perilli, S., Taniguchi, M., Morita, M.T., Aoyama, T., Costantino, P., and Sabatini, S. (2008). A genetic framework for the control of cell division and differentiation in the root meristem. Science *322*, 1380-1384.

DeLong, A., Mockaitis, K., and Christensen, S. (2002). Protein phosphorylation in the delivery of and response to auxin signals. Plant Mol Biol *49*, 285-303.

Deruere, J., Jackson, K., Garbers, C., Soll, D., and Delong, A. (1999). The RCN1-encoded A subunit of protein phosphatase 2A increases phosphatase activity in vivo. Plant J *20*, 389-399.

Dharmasiri, N., Dharmasiri, S., and Estelle, M. (2005). The F-box protein TIR1 is an auxin receptor. Nature *435*, 441-445.

Dharmasiri, S., Swarup, R., Mockaitis, K., Dharmasiri, N., Singh, S.K., Kowalchyk, M., Marchant, A., Mills, S., Sandberg, G., Bennett, M.J., *et al.* (2006). AXR4 is required for localization of the auxin influx facilitator AUX1. Science *312*, 1218-1220.

Dhonukshe, P., Aniento, F., Hwang, I., Robinson, D.G., Mravec, J., Stierhof, Y.D., and Friml, J. (2007). Clathrin-mediated constitutive endocytosis of PIN auxin efflux carriers in Arabidopsis. Curr Biol *17*, 520-527.

Dhonukshe, P., Grigoriev, I., Fischer, R., Tominaga, M., Robinson, D.G., Hasek, J., Paciorek, T., Petrasek, J., Seifertova, D., Tejos, R., *et al.* (2008). Auxin transport inhibitors impair vesicle motility and actin cytoskeleton dynamics in diverse eukaryotes. Proc Natl Acad Sci U S A *105*, 4489-4494.

Dubrovsky, J.G., Sauer, M., Napsucialy-Mendivil, S., Ivanchenko, M.G., Friml, J., Shishkova, S., Celenza, J., and Benkova, E. (2008). Auxin acts as a local morphogenetic trigger to specify lateral root founder cells. Proc Natl Acad Sci U S A *105*, 8790-8794.

Dudler, R., and Hertig, C. (1992). Structure of an mdr-like gene from Arabidopsis thaliana. Evolutionary implications. J Biol Chem *267*, 5882-5888.

Dudler, R., and Sidler, M. (1998). Arabidopsis MDR genes: molecular cloning and protein chemical aspects. Methods Enzymol *292*, 162-173.

Edlich, F., Maestre-Martinez, M., Jarczowski, F., Weiwad, M., Moutty, M.C., Malesevic, M., Jahreis, G., Fischer, G., and Lucke, C. (2007). A novel calmodulin-Ca2+ target recognition activates the Bcl-2 regulator FKBP38. J Biol Chem *282*, 36496-36504.

Esmon, C.A., Pedmale, U.V., and Liscum, E. (2005). Plant tropisms: providing the power of movement to a sessile organism. Int J Dev Biol *49*, 665-674.

Feraru, E., and Friml, J. (2008). PIN polar targeting. Plant Physiol *147*, 1553-1559.

Ferte, J., Kuhnel, J.M., Chapuis, G., Rolland, Y., Lewin, G., and Schwaller, M.A. (1999). Flavonoid-related modulators of multidrug resistance: synthesis, pharmacological activity, and structure-activity relationships. J Med Chem *42*, 478-489.

Feucht, W., Dithmar, H., and Polster, J. (2004). Nuclei of tea flowers as targets for flavanols. Plant Biol (Stuttg) *6*, 696-701.

Feucht, W., and Polster, J. (2001). Nuclei of plants as a sink for flavanols. Z Naturforsch C *56*, 479-481.

Friedrichsen, D.M., Joazeiro, C.A., Li, J., Hunter, T., and Chory, J. (2000). Brassinosteroid-insensitive-1 is a ubiquitously expressed leucine-rich repeat receptor serine/threonine kinase. Plant Physiol *123*, 1247-1256.

Friml, J. (2003). Auxin transport - shaping the plant. Curr Opin Plant Biol *6*, 7-12.

Friml, J., Benkova, E., Blilou, I., Wisniewska, J., Hamann, T., Ljung, K., Woody, S., Sandberg, G., Scheres, B., Jurgens, G.*, et al.* (2002a). AtPIN4 mediates sink-driven auxin gradients and root patterning in Arabidopsis. Cell *108*, 661-673.

Friml, J., and Palme, K. (2002). Polar auxin transport--old questions and new concepts? Plant Mol Biol *49*, 273-284.

Friml, J., Vieten, A., Sauer, M., Weijers, D., Schwarz, H., Hamann, T., Offringa, R., and Jurgens, G. (2003). Efflux-dependent auxin gradients establish the apical-basal axis of Arabidopsis. Nature *426*, 147-153.

Friml, J., Wisniewska, J., Benkova, E., Mendgen, K., and Palme, K. (2002b). Lateral relocation of auxin efflux regulator PIN3 mediates tropism in Arabidopsis. Nature *415*, 806-809.

Friml, J., Yang, X., Michniewicz, M., Weijers, D., Quint, A., Tietz, O., Benjamins, R., Ouwerkerk, P.B., Ljung, K., Sandberg, G.*, et al.* (2004). A PINOID-dependent binary switch in apical-basal PIN polar targeting directs auxin efflux. Science *306*, 862-865.

Fu, D., and Roufogalis, B.D. (2007). Actin disruption inhibits endosomal traffic of P-glycoprotein-EGFP and resistance to daunorubicin accumulation. Am J Physiol Cell Physiol *292*, C1543-1552.

Furutani, M., Kajiwara, T., Kato, T., Treml, B.S., Stockum, C., Torres-Ruiz, R.A., and Tasaka, M. (2007). The gene MACCHI-BOU 4/ENHANCER OF PINOID encodes a NPH3-like protein and reveals similarities between organogenesis and phototropism at the molecular level. Development *134*, 3849-3859.

Galvan-Ampudia, C.S., and Offringa, R. (2007). Plant evolution: AGC kinases tell the auxin tale. Trends Plant Sci *12*, 541-547.

Galweiler, L., Conlan, R.S., Mader, P., Palme, K., and Moore, I. (2000). Technical advance: the DNA-binding activity of gal4 is inhibited by methylation of the gal4 binding site in plant chromatin. Plant J *23*, 143-157.

Galweiler, L., Guan, C., Muller, A., Wisman, E., Mendgen, K., Yephremov, A., and Palme, K. (1998). Regulation of polar auxin transport by AtPIN1 in Arabidopsis vascular tissue. Science *282*, 2226-2230.

Gamet-Payrastre, L., Manenti, S., Gratacap, M.P., Tulliez, J., Chap, H., and Payrastre, B. (1999). Flavonoids and the inhibition of PKC and PI 3-kinase. Gen Pharmacol *32*, 279-286.

Garbers, C., DeLong, A., Deruere, J., Bernasconi, P., and Soll, D. (1996). A mutation in protein phosphatase 2A regulatory subunit A affects auxin transport in Arabidopsis. EMBO J *15*, 2115-2124.

Geisler, M., and Bailly, A. (2007). Tete-a-tete: the function of FKBPs in plant development. Trends Plant Sci *12*, 465-473.

Geisler, M., Blakeslee, J.J., Bouchard, R., Lee, O.R., Vincenzetti, V., Bandyopadhyay, A., Titapiwatanakun, B., Peer, W.A., Bailly, A., Richards, E.L*., et al.* (2005). Cellular efflux of auxin catalyzed by the Arabidopsis MDR/PGP transporter AtPGP1. Plant J *44*, 179-194.

Geisler, M., Girin, M., Brandt, S., Vincenzetti, V., Plaza, S., Paris, N., Kobae, Y., Maeshima, M., Billion, K., Kolukisaoglu, U.H*., et al.* (2004). Arabidopsis immunophilin-like TWD1 functionally interacts with vacuolar ABC transporters. Mol Biol Cell *15*, 3393-3405.

Geisler, M., Kolukisaoglu, H.U., Bouchard, R., Billion, K., Berger, J., Saal, B., Frangne, N., Koncz-Kalman, Z., Koncz, C., Dudler, R*., et al.* (2003). TWISTED DWARF1, a unique plasma membrane-anchored immunophilin-like protein, interacts with Arabidopsis multidrug resistance-like transporters AtPGP1 and AtPGP19. Mol Biol Cell *14*, 4238-4249.

Geisler, M., and Murphy, A.S. (2006). The ABC of auxin transport: the role of p-glycoproteins in plant development. FEBS Lett *580*, 1094-1102.

Geldner, N., Friml, J., Stierhof, Y.D., Jurgens, G., and Palme, K. (2001). Auxin transport inhibitors block PIN1 cycling and vesicle trafficking. Nature *413*, 425-428.

Germann, U.A. (1996). P-glycoprotein--a mediator of multidrug resistance in tumour cells. Eur J Cancer *32A*, 927-944.

Germann, U.A., Chambers, T.C., Ambudkar, S.V., Licht, T., Cardarelli, C.O., Pastan, I., and Gottesman, M.M. (1996). Characterization of phosphorylation-defective mutants of human P-glycoprotein expressed in mammalian cells. J Biol Chem *271*, 1708-1716.

Glavy, J.S., Horwitz, S.B., and Orr, G.A. (1997). Identification of the in vivo phosphorylation sites for acidic-directed kinases in murine mdr1b P-glycoprotein. J Biol Chem *272*, 5909-5914.

Goodfellow, H.R., Sardini, A., Ruetz, S., Callaghan, R., Gros, P., McNaughton, P.A., and Higgins, C.F. (1996). Protein kinase C-mediated phosphorylation does not regulate drug transport by the human multidrug resistance P-glycoprotein. J Biol Chem *271*, 13668-13674.

Grunewald, W., Cannoot, B., Friml, J., and Gheysen, G. (2009). Parasitic nematodes modulate PIN-mediated auxin transport to facilitate infection. PLoS Pathog *5*, e1000266.

Gutzeit, H.O., Henker, Y., Kind, B., and Franz, A. (2004). Specific interactions of quercetin and other flavonoids with target proteins are revealed by elicited fluorescence. Biochem Biophys Res Commun *318*, 490-495.

Hagen, G., and Guilfoyle, T. (2002). Auxin-responsive gene expression: genes, promoters and regulatory factors. Plant Mol Biol *49*, 373-385.

Hardtke, C.S., Ckurshumova, W., Vidaurre, D.P., Singh, S.A., Stamatiou, G., Tiwari, S.B., Hagen, G., Guilfoyle, T.J., and Berleth, T. (2004). Overlapping and non-redundant functions of the Arabidopsis auxin response factors MONOPTEROS and NONPHOTOTROPIC HYPOCOTYL 4. Development *131*, 1089-1100.

Herbert, J.M., Augereau, J.M., Gleye, J., and Maffrand, J.P. (1990). Chelerythrine is a potent and specific inhibitor of protein kinase C. Biochem Biophys Res Commun *172*, 993-999.

Hertel, R., Thomson, K.S., and Russo, V.E.A. (1972). In-vitro auxin binding to particulate cell fractions from corn coleoptiles. Planta (Berl) *107*, 325--340.

Hossel, D., Schmeiser, C., and Hertel, R. (2005). Specificity patterns indicate that auxin exporters and receptors are the same proteins. Plant Biol (Stuttg) *7*, 41-48.

Hrycyna, C.A., Airan, L.E., Germann, U.A., Ambudkar, S.V., Pastan, I., and Gottesman, M.M. (1998). Structural flexibility of the linker region of human P-glycoprotein permits ATP hydrolysis and drug transport. Biochemistry *37*, 13660-13673.

Idriss, H.T., Hannun, Y.A., Boulpaep, E., and Basavappa, S. (2000). Regulation of volume-activated chloride channels by P-glycoprotein: phosphorylation has the final say! J Physiol *524 Pt 3*, 629-636.

Ikeda, Y., Men, S., Fischer, U., Stepanova, A.N., Alonso, J.M., Ljung, K., and Grebe, M. (2009). Local auxin biosynthesis modulates gradient-directed planar polarity in Arabidopsis. Nat Cell Biol *11*, 731-738.

Kamphausen, T., Fanghanel, J., Neumann, D., Schulz, B., and Rahfeld, J.U. (2002). Characterization of Arabidopsis thaliana AtFKBP42 that is membrane-bound and interacts with Hsp90. Plant J *32*, 263-276.

Katekar, G.F., and Geissler, A.E. (1975). Auxin Transport Inhibitors: Fluorescein and Related Compounds. Plant Physiol *56*, 645-646.

Kepinski, S., and Leyser, O. (2005). The Arabidopsis F-box protein TIR1 is an auxin receptor. Nature *435*, 446-451.

Kim, H.M., Oh, G.T., Hong, D.H., Kim, M.S., Kang, J.S., Park, S.M., and Han, S.B. (1997). MDR-1 gene expression is a minor factor in determining the multidrug resistance phenotype of MCF7/ADR and KB-V1 cells. FEBS Lett *412*, 201-206.

Kim, J.I., Sharkhuu, A., Jin, J.B., Li, P., Jeong, J.C., Baek, D., Lee, S.Y., Blakeslee, J.J., Murphy, A.S., Bohnert, H.J.*, et al.* (2007a). yucca6, a dominant mutation in Arabidopsis, affects auxin accumulation and auxin-related phenotypes. Plant Physiol *145*, 722-735.

Kim, T.W., Lee, S.M., Joo, S.H., Yun, H.S., Lee, Y., Kaufman, P.B., Kirakosyan, A., Kim, S.H., Nam, K.H., Lee, J.S.*, et al.* (2007b). Elongation and gravitropic responses of Arabidopsis roots are regulated by brassinolide and IAA. Plant Cell Environ *30*, 679-689.

Kleine-Vehn, J., and Friml, J. (2008). Polar targeting and endocytic recycling in auxin-dependent plant development. Annu Rev Cell Dev Biol *24*, 447-473.

Kleine-Vehn, J., Leitner, J., Zwiewka, M., Sauer, M., Abas, L., Luschnig, C., and Friml, J. (2008). Differential degradation of PIN2 auxin efflux carrier by retromer-dependent vacuolar targeting. Proc Natl Acad Sci U S A *105*, 17812-17817.

Lau, S., Jurgens, G., and De Smet, I. (2008). The evolving complexity of the auxin pathway. Plant Cell *20*, 1738-1746.

Laxmi, A., Pan, J., Morsy, M., and Chen, R. (2008). Light plays an essential role in intracellular distribution of auxin efflux carrier PIN2 in Arabidopsis thaliana. PLoS ONE *3*, e1510.

Lee, S.H., and Cho, H.T. (2006). PINOID positively regulates auxin efflux in Arabidopsis root hair cells and tobacco cells. Plant Cell *18*, 1604-1616.

Lee, S.J., Bohm, A., Krug, M., and Boos, W. (2007). The ABC of binding-protein-dependent transport in Archaea. Trends Microbiol *15*, 389-397.

Lee, S.K., Qing, W.G., Mar, W., Luyengi, L., Mehta, R.G., Kawanishi, K., Fong, H.H., Beecher, C.W., Kinghorn, A.D., and Pezzuto, J.M. (1998). Angoline and chelerythrine, benzophenanthridine alkaloids that do not inhibit protein kinase C. J Biol Chem *273*, 19829-19833.

Lewis, D.R., Miller, N.D., Splitt, B.L., Wu, G., and Spalding, E.P. (2007). Separating the roles of acropetal and basipetal auxin transport on gravitropism with mutations in two Arabidopsis multidrug resistance-like ABC transporter genes. Plant Cell *19*, 1838-1850.

Limtrakul, P., Khantamat, O., and Pintha, K. (2005). Inhibition of P-glycoprotein function and expression by kaempferol and quercetin. J Chemother *17*, 86-95.

Lin, R., and Wang, H. (2005). Two homologous ATP-binding cassette transporter proteins, AtMDR1 and AtPGP1, regulate Arabidopsis photomorphogenesis and root development by mediating polar auxin transport. Plant Physiol *138*, 949-964.

Lomax, T.L., Muday, G.K., and Rubery, P.H. (1995). Auxin transport. In Plant Hormones: Physiology, Biochemistry and Molecular Biology, P.J. Davies, ed. (Dordrecht, Netherlands, Kluwer), pp. 509-530.

Luschnig, C., Gaxiola, R.A., Grisafi, P., and Fink, G.R. (1998). EIR1, a root-specific protein involved in auxin transport, is required for gravitropism in Arabidopsis thaliana. Genes Dev *12*, 2175-2187.

MacKintosh, C., and MacKintosh, R.W. (1994). Inhibitors of protein kinases and phosphatases. Trends Biochem Sci *19*, 444-448.

Malenica, N., Abas, L., Benjamins, R., Kitakura, S., Sigmund, H.F., Jun, K.S., Hauser, M.T., Friml, J., and Luschnig, C. (2007). MODULATOR OF PIN genes control steady-state levels of Arabidopsis PIN proteins. Plant J *51*, 537-550.

Marchant, A., Bhalerao, R., Casimiro, I., Eklof, J., Casero, P.J., Bennett, M., and Sandberg, G. (2002). AUX1 promotes lateral root formation by facilitating indole-3-acetic acid distribution between sink and source tissues in the Arabidopsis seedling. Plant Cell *14*, 589-597.

Martinoia, E., Klein, M., Geisler, M., Bovet, L., Forestier, C., Kolukisaoglu, U., Muller-Rober, B., and Schulz, B. (2002). Multifunctionality of plant ABC transporters--more than just detoxifiers. Planta *214*, 345-355.

McSteen, P., Malcomber, S., Skirpan, A., Lunde, C., Wu, X., Kellogg, E., and Hake, S. (2007). Barren inflorescence2 Encodes a co-ortholog of the PINOID serine/threonine kinase and is required for organogenesis during inflorescence and vegetative development in maize. Plant Physiol 144, 1000-1011.

Mense, M., Vergani, P., White, D.M., Altberg, G., Nairn, A.C., and Gadsby, D.C. (2006). In vivo phosphorylation of CFTR promotes formation of a nucleotide-binding domain heterodimer. EMBO J *25*, 4728-4739.

Merks, R.M., Van de Peer, Y., Inze, D., and Beemster, G.T. (2007). Canalization without flux sensors: a traveling-wave hypothesis. Trends Plant Sci *12*, 384-390.

Michniewicz, M., Zago, M.K., Abas, L., Weijers, D., Schweighofer, A., Meskiene, I., Heisler, M.G., Ohno, C., Zhang, J., Huang, F.*, et al.* (2007). Antagonistic regulation of PIN phosphorylation by PP2A and PINOID directs auxin flux. Cell *130*, 1044-1056.

Mizushina, Y., Ikuta, A., Endoh, K., Oshige, M., Kasai, N., Kamiya, K., Satake, T., Takazawa, H., Morita, H., Tomiyasu, H.*, et al.* (2003). Inhibition of DNA polymerases and DNA topoisomerase II by triterpenes produced by plant callus. Biochem Biophys Res Commun *305*, 365-373.

Morita, Y., and Kyozuka, J. (2007). Characterization of OsPID, the rice ortholog of PINOID, and its possible involvement in the control of polar auxin transport. Plant Cell Physiol **48,** 540-549.

Mravec, J., Kubes, M., Bielach, A., Gaykova, V., Petrasek, J., Skupa, P., Chand, S., Benkova, E., Zazimalova, E., and Friml, J. (2008). Interaction of PIN and PGP transport mechanisms in auxin distribution-dependent development. Development *135*, 3345-3354.

Mravec, J., Skupa, P., Bailly, A., Hoyerova, K., Krecek, P., Bielach, A., Petrasek, J., Zhang, J., Gaykova, V., Stierhof, Y.D.*, et al.* (2009). Subcellular homeostasis of phytohormone auxin is mediated by the ER-localized PIN5 transporter. Nature *459*, 1136-1140.

Muday, G.K., and DeLong, A. (2001). Polar auxin transport: controlling where and how much. Trends Plant Sci *6*, 535-542.

Multani, D.S., Briggs, S.P., Chamberlin, M.A., Blakeslee, J.J., Murphy, A.S., and Johal, G.S. (2003). Loss of an MDR transporter in compact stalks of maize br2 and sorghum dw3 mutants. Science *302*, 81-84.

Murphy, A., Peer, W.A., and Taiz, L. (2000). Regulation of auxin transport by aminopeptidases and endogenous flavonoids. Planta *211*, 315-324.

Murphy, A.S., Hoogner, K.R., Peer, W.A., and Taiz, L. (2002). Identification, purification, and molecular cloning of N-1-naphthylphthalmic acid-binding plasma membrane-associated aminopeptidases from Arabidopsis. Plant Physiol *128*, 935-950.

Nakamura, A., Higuchi, K., Goda, H., Fujiwara, M.T., Sawa, S., Koshiba, T., Shimada, Y., and Yoshida, S. (2003). Brassinolide induces IAA5, IAA19, and DR5, a synthetic auxin response element in Arabidopsis, implying a cross talk point of brassinosteroid and auxin signaling. Plant Physiol *133*, 1843-1853.

Nemhauser, J.L., Mockler, T.C., and Chory, J. (2004). Interdependency of brassinosteroid and auxin signaling in Arabidopsis. PLoS Biol *2*, E258.

Noh, B., Murphy, A.S., and Spalding, E.P. (2001). Multidrug resistance-like genes of Arabidopsis required for auxin transport and auxin-mediated development. Plant Cell *13*, 2441-2454.

Nuhse, T.S., Stensballe, A., Jensen, O.N., and Peck, S.C. (2004). Phosphoproteomics of the Arabidopsis plasma membrane and a new phosphorylation site database. Plant Cell *16*, 2394-2405.

Okada, K., Ueda, J., Komaki, M.K., Bell, C.J., and Shimura, Y. (1991). Requirement of the Auxin Polar Transport System in Early Stages of Arabidopsis Floral Bud Formation. Plant Cell *3*, 677-684.

Okamoto, Tanaka, and Sakai. (1991). Molecular cloning and analysis of the c DNA for an auxin-regulated calmodulin gene. Plant Cell Physiol. 36, 1531-1539.

Orlowski, S., Martin, S., and Escargueil, A. (2006). P-glycoprotein and 'lipid rafts': some ambiguous mutual relationships (floating on them, building them or meeting them by chance?). Cell Mol Life Sci *63*, 1038-1059.

Orr, G.A., Han, E.K., Browne, P.C., Nieves, E., O'Connor, B.M., Yang, C.P., and Horwitz, S.B. (1993). Identification of the major phosphorylation domain of murine mdr1b P-glycoprotein. Analysis of the protein kinase A and protein kinase C phosphorylation sites. J Biol Chem *268*, 25054-25062.

Ostin, A., Ilic, N., and Cohen, J.D. (1999). An in vitro system from maize seedlings for tryptophan-independent indole-3-acetic acid biosynthesis. Plant Physiol *119*, 173-178.

Ottenschlager, I., Wolff, P., Wolverton, C., Bhalerao, R.P., Sandberg, G., Ishikawa, H., Evans, M., and Palme, K. (2003). Gravity-regulated differential auxin transport from columella to lateral root cap cells. Proc Natl Acad Sci U S A *100*, 2987-2991.

Paciorek, T., Zazimalova, E., Ruthardt, N., Petrasek, J., Stierhof, Y.D., Kleine-Vehn, J., Morris, D.A., Emans, N., Jurgens, G., Geldner, N.*, et al.* (2005). Auxin inhibits endocytosis and promotes its own efflux from cells. Nature *435*, 1251-1256.

Pagnussat, G.C., Alandete-Saez, M., Bowman, J.L., and Sundaresan, V. (2009). Auxin-dependent patterning and gamete specification in the Arabidopsis female gametophyte. Science *324*, 1684-1689.

Palme, K., Dovzhenko, A., and Ditengou, F.A. (2006). Auxin transport and gravitational research: perspectives. Protoplasma *229*, 175-181.

Palme, K., and Nagy, F. (2008). A new gene for auxin synthesis. Cell *133*, 31-32.

Parry, G., Delbarre, A., Marchant, A., Swarup, R., Napier, R., Perrot-Rechenmann, C., and Bennett, M.J. (2001). Novel auxin transport inhibitors phenocopy the auxin influx carrier mutation aux1. Plant J *25*, 399-406.

Peer, W.A., Bandyopadhyay, A., Blakeslee, J.J., Makam, S.N., Chen, R.J., Masson, P.H., and Murphy, A.S. (2004). Variation in expression and protein localization of the PIN family of auxin efflux

facilitator proteins in flavonoid mutants with altered auxin transport in Arabidopsis thaliana. Plant Cell *16*, 1898-1911.

Peer, W.A., Brown, D.E., Tague, B.W., Muday, G.K., Taiz, L., and Murphy, A.S. (2001). Flavonoid accumulation patterns of transparent testa mutants of arabidopsis. Plant Physiol *126*, 536-548.

Peer, W.A., and Murphy, A.S. (2007). Flavonoids and auxin transport: modulators or regulators? Trends Plant Sci *12*, 556-563.

Pennazio, S. (2002). The discovery of the chemical nature of the plant hormone auxin. Riv Biol *95*, 289-308.

Perez-Perez, J.M., Ponce, M.R., and Micol, J.L. (2004). The ULTRACURVATA2 gene of Arabidopsis encodes an FK506-binding protein involved in auxin and brassinosteroid signaling. Plant Physiol *134*, 101-117.

Pernisova, M., Klima, P., Horak, J., Valkova, M., Malbeck, J., Soucek, P., Reichman, P., Hoyerova, K., Dubova, J., Friml, J*., et al.* (2009). Cytokinins modulate auxin-induced organogenesis in plants via regulation of the auxin efflux. Proc Natl Acad Sci U S A *106*, 3609-3614.

Petersson, S.V., Johansson, A.I., Kowalczyk, M., Makoveychuk, A., Wang, J.Y., Moritz, T., Grebe, M., Benfey, P.N., Sandberg, G., and Ljung, K. (2009). An Auxin Gradient and Maximum in the Arabidopsis Root Apex Shown by High-Resolution Cell-Specific Analysis of IAA Distribution and Synthesis. Plant Cell.

Petrasek, J., Cerna, A., Schwarzerova, K., Elckner, M., Morris, D.A., and Zazimalova, E. (2003). Do phytotropins inhibit auxin efflux by impairing vesicle traffic? Plant Physiol *131*, 254-263.

Petrasek, J., and Friml, J. (2009). Auxin transport routes in plant development. Development 136, 2675-2688.

Pickett, F.B., Wilson, A.K., and Estelle, M. (1990). The aux1 Mutation of Arabidopsis Confers Both Auxin and Ethylene Resistance. Plant Physiol *94*, 1462-1466.

Pufky, J., Qiu, Y., Rao, M.V., Hurban, P., and Jones, A.M. (2003). The auxin-induced transcriptome for etiolated Arabidopsis seedlings using a structure/function approach. Funct Integr Genomics *3*, 135-143.

Quint, M., and Gray, W.M. (2006). Auxin signaling. Curr Opin Plant Biol *9*, 448-453.

Ramadass, P., Meerarani, P., Toborek, M., Robertson, L.W., and Hennig, B. (2003). Dietary flavonoids modulate PCB-induced oxidative stress, CYP1A1 induction, and AhR-DNA binding activity in vascular endothelial cells. Toxicol Sci *76*, 212-219.

Ramaen, O., Sizun, C., Pamlard, O., Jacquet, E., and Lallemand, J.Y. (2005). Attempts to characterize the NBD heterodimer of MRP1: transient complex formation involves Gly771 of the ABC signature sequence but does not enhance the intrinsic ATPase activity. Biochem J *391*, 481-490.

Rampey, R.A., Woodward, A.W., Hobbs, B.N., Tierney, M.P., Lahner, B., Salt, D.E., and Bartel, B. (2006). An Arabidopsis basic helix-loop-helix leucine zipper protein modulates metal homeostasis and auxin conjugate responsiveness. Genetics *174*, 1841-1857.

Rashotte, A.M., DeLong, A., and Muday, G.K. (2001). Genetic and chemical reductions in protein phosphatase activity alter auxin transport, gravity response, and lateral root growth. Plant Cell *13*, 1683-1697.

Ratnasinghe, D., Phang, J.M., and Yeh, G.C. (1998). Differential expression and activity of phosphatases and protein kinases in adriamycin sensitive and resistant human breast cancer MCF-7 cells. Int J Oncol *13*, 79-84.

Rice-Evans, C. (2001). Flavonoid antioxidants. Curr Med Chem *8*, 797-807.

Rich, D.P., Gregory, R.J., Anderson, M.P., Manavalan, P., Smith, A.E., and Welsh, M.J. (1991). Effect of deleting the R domain on CFTR-generated chloride channels. Science *253*, 205-207.

Robert, H.S., and Friml, J. (2009). Auxin and other signals on the move in plants. Nat Chem Biol *5*, 325-332.

Rojas-Pierce, M., Titapiwatanakun, B., Sohn, E.J., Fang, F., Larive, C.K., Blakeslee, J., Cheng, Y., Cutler, S.R., Peer, W.A., Murphy, A.S., (2007). Arabidopsis P-glycoprotein19 participates in the inhibition of gravitropism by gravacin. Chem Biol *14*, 1366-1376.

Rubery, P.H., and Sheldrake, A.R. (1973). Effect of pH and surface charge on cell uptake of auxin. Nat New Biol *244*, 285-288.

Ruegger, M., Dewey, E., Hobbie, L., Brown, D., Bernasconi, P., Turner, J., Muday, G. and Estelle, M. (1997). Reduced naphthylphthalamic acid binding in the *tir3* mutant of Arabidopsis is associated with a reduction in polar auxin transport and diverse morphological defects. Plant Cell 9, 745-757.

Sachs, S. (1865). Handbuch der Experimentalphysiologie der Pflanzen.

Sachs, T. (1986). Cellular interactions in tissue and organ development. Symp Soc Exp Biol *40*, 181-210.

Samaj, J., Ovecka, M., Hlavacka, A., Lecourieux, F., Meskiene, I., Lichtscheidl, I., Lenart, P., Salaj, J., Volkmann, D., Bogre, L.*, et al.* (2002). Involvement of the mitogen-activated protein kinase SIMK in regulation of root hair tip growth. EMBO J *21*, 3296-3306.

Santelia, D., Henrichs, S., Vincenzetti, V., Sauer, M., Bigler, L., Klein, M., Bailly, A., Lee, Y., Friml, J., Geisler, M.*, et al.* (2008). Flavonoids redirect PIN-mediated polar auxin fluxes during root gravitropic responses. J Biol Chem *283*, 31218-31226.

Santelia, D., Vincenzetti, V., Azzarello, E., Bovet, L., Fukao, Y., Duchtig, P., Mancuso, S., Martinoia, E., and Geisler, M. (2005). MDR-like ABC transporter AtPGP4 is involved in auxin-mediated lateral root and root hair development. FEBS Lett *579*, 5399-5406.

Santner, A.A., and Watson, J.C. (2006). The WAG1 and WAG2 protein kinases negatively regulate root waving in Arabidopsis. Plant J *45*, 752-764.

Sauer, M., Balla, J., Luschnig, C., Wisniewska J., Reinöhl, V., Friml, J. and Benkova E. (2006). Canalization of auxin flow by Aux/IAA-ARF-dependent feedback regulation of PIN polarity. Development 20: 2902-2911.

Saslowsky, D.E., Warek, U., and Winkel, B.S. (2005). Nuclear localization of flavonoid enzymes in Arabidopsis. J Biol Chem *280*, 23735-23740.

Scarpella, E., Marcos, D., Friml, J., and Berleth, T. (2006). Control of leaf vascular patterning by polar auxin transport. Genes Dev *20*, 1015-1027.

Scheidt, H.A., Vogel, A., Eckhoff, A., Koenig, B.W., and Huster, D. (2007). Solid-state NMR characterization of the putative membrane anchor of TWD1 from Arabidopsis thaliana. Eur Biophys J *36*, 393-404.

Senderowicz, A.M. (2000). Small molecule modulators of cyclin-dependent kinases for cancer therapy. Oncogene *19*, 6600-6606.

Senior, A.E., al-Shawi, M.K., and Urbatsch, I.L. (1995). The catalytic cycle of P-glycoprotein. FEBS Lett *377*, 285-289.

Shin, H., Shin, H.S., Guo, Z., Blancaflor, E.B., Masson, P.H., and Chen, R. (2005). Complex regulation of Arabidopsis AGR1/PIN2-mediated root gravitropic response and basipetal auxin transport by cantharidin-sensitive protein phosphatases. Plant J *42*, 188-200.

Sidler, M., Hassa, P., Hasan, S., Ringli, C., and Dudler, R. (1998). Involvement of an ABC transporter in a developmental pathway regulating hypocotyl cell elongation in the light. Plant Cell *10*, 1623-1636.

Skirpan, A., Culler, A.H., Gallavotti, A., Jackson, D., Cohen, J.D., and McSteen, P. (2009). BARREN INFLORESCENCE2 interaction with ZmPIN1a suggests a role in auxin transport during maize inflorescence development. Plant Cell Physiol.

Sistrunk, M.L., Antosiewicz, D.M., Purugganan, M.M., and Braam, J. (1994). Arabidopsis TCH3 encodes a novel Ca2+ binding protein and shows environmentally induced and tissue-specific regulation. Plant Cell *6*, 1553-1565.

Sorefan, K., Girin, T., Liljegren, S.J., Ljung, K., Robles, P., Galvan-Ampudia, C.S., Offringa, R., Friml, J., Yanofsky, M.F., and Ostergaard, L. (2009). A regulated auxin minimum is required for seed dispersal in Arabidopsis. Nature *459*, 583-586.

Spoel, S.H., and Dong, X. (2008). Making sense of hormone crosstalk during plant immune responses. Cell Host Microbe *3*, 348-351.

Staswick, P.E. (2009). The tryptophan conjugates of jasmonic and indole-3-acetic acids are endogenous auxin inhibitors. Plant Physiol *150*, 1310-1321.

Staswick, P.E., Serban, B., Rowe, M., Tiryaki, I., Maldonado, M.T., Maldonado, M.C., and Suza, W. (2005). Characterization of an Arabidopsis enzyme family that conjugates amino acids to indole-3-acetic acid. Plant Cell *17*, 616-627.

Stepanova, A.N., Robertson-Hoyt, J., Yun, J., Benavente, L.M., Xie, D.Y., Dolezal, K., Schlereth, A., Jurgens, G., and Alonso, J.M. (2008). TAA1-mediated auxin biosynthesis is essential for hormone crosstalk and plant development. Cell *133*, 177-191.

Sukumar, P., Edwards, K.S., Rahman, A., Delong, A., and Muday, G.K. (2009). PINOID kinase regulates root gravitropism through modulation of PIN2-dependent basipetal auxin transport in Arabidopsis. Plant Physiol *150*, 722-735.

Surpin, M., Rojas-Pierce, M., Carter, C., Hicks, G.R., Vasquez, J., and Raikhel, N.V. (2005). The power of chemical genomics to study the link between endomembrane system components and the gravitropic response. Proc Natl Acad Sci U S A *102*, 4902-4907.

Swarup, K., Benkova, E., Swarup, R., Casimiro, I., Peret, B., Yang, Y., Parry, G., Nielsen, E., De Smet, I., Vanneste, S.*, et al.* (2008). The auxin influx carrier LAX3 promotes lateral root emergence. Nat Cell Biol *10*, 946-954.

Swarup, R., Friml, J., Marchant, A., Ljung, K., Sandberg, G., Palme, K., and Bennett, M. (2001). Localization of the auxin permease AUX1 suggests two functionally distinct hormone transport pathways operate in the Arabidopsis root apex. Genes Dev *15*, 2648-2653.

Swarup, R., Kramer, E.M., Perry, P., Knox, K., Leyser, H.M., Haseloff, J., Beemster, G.T., Bhalerao, R., and Bennett, M.J. (2005). Root gravitropism requires lateral root cap and epidermal cells for transport and response to a mobile auxin signal. Nat Cell Biol *7*, 1057-1065.

Szabo, K., Bakos, E., Welker, E., Muller, M., Goodfellow, H.R., Higgins, C.F., Varadi, A., and Sarkadi, B. (1997). Phosphorylation site mutations in the human multidrug transporter modulate its drug-stimulated ATPase activity. J Biol Chem *272*, 23165-23171.

Tao, Y., Ferrer, J.L., Ljung, K., Pojer, F., Hong, F., Long, J.A., Li, L., Moreno, J.E., Bowman, M.E., Ivans, L.J.*, et al.* (2008). Rapid synthesis of auxin via a new tryptophan-dependent pathway is required for shade avoidance in plants. Cell *133*, 164-176.

Terasaka, K., Blakeslee, J.J., Titapiwatanakun, B., Peer, W.A., Bandyopadhyay, A., Makam, S.N., Lee, O.R., Richards, E.L., Murphy, A.S., Sato, F.*, et al.* (2005). PGP4, an ATP binding cassette P-glycoprotein, catalyzes auxin transport in Arabidopsis thaliana roots. Plant Cell *17*, 2922-2939.

Thimann, K.V. (1938). Hormones and the Analysis of Growth. Plant Physiol *13*, 437-449.

Titapiwatanakun, B., Blakeslee, J.J., Bandyopadhyay, A., Yang, H., Mravec, J., Sauer, M., Cheng, Y., Adamec, J., Nagashima, A., Geisler, M.*, et al.* (2009). ABCB19/PGP19 stabilises PIN1 in membrane microdomains in Arabidopsis. Plant J *57*, 27-44.

Toyota, M., Furuichi, T., Tatsumi, H., and Sokabe, M. (2008). Cytoplasmic calcium increases in response to changes in the gravity vector in hypocotyls and petioles of Arabidopsis seedlings. Plant Physiol *146*, 505-514.

Ulmasov, T., Murfett, J., Hagen, G., and Guilfoyle, T.J. (1997). Aux/IAA proteins repress expression of reporter genes containing natural and highly active synthetic auxin response elements. Plant Cell *9*, 1963-1971.

Vanoye, C.G., Altenberg, G.A., and Reuss, L. (1997). P-glycoprotein is not a swelling-activated Cl-channel; possible role as a Cl- channel regulator. J Physiol *502 (Pt 2)*, 249-258.

Vanneste, S., and Friml, J. (2009). Auxin: a trigger for change in plant development. Cell 136, 1005-1016.

Verrier, P.J., Bird, D., Burla, B., Dassa, E., Forestier, C., Geisler, M., Klein, M., Kolukisaoglu, U., Lee, Y., Martinoia, E.*, et al.* (2008). Plant ABC proteins--a unified nomenclature and updated inventory. Trends Plant Sci *13*, 151-159.

Vieten, A., Vanneste, S., Wisniewska, J., Benkova, E., Benjamins, R., Beeckman, T., Luschnig, C., and Friml, J. (2005). Functional redundancy of PIN proteins is accompanied by auxin-dependent cross-regulation of PIN expression. Development *132*, 4521-4531.

Walle, T., Vincent, T.S., and Walle, U.K. (2003). Evidence of covalent binding of the dietary flavonoid quercetin to DNA and protein in human intestinal and hepatic cells. Biochem Pharmacol *65*, 1603-1610.

Wietzke, J.A., and Welsh, J. (2003). Phytoestrogen regulation of a Vitamin D3 receptor promoter and 1,25-dihydroxyvitamin D3 actions in human breast cancer cells. J Steroid Biochem Mol Biol *84*, 149-157.

Windsor, B., Roux, S.J., and Lloyd, A. (2003). Multiherbicide tolerance conferred by AtPgp1 and apyrase overexpression in Arabidopsis thaliana. Nat Biotechnol *21*, 428-433.

Winkel-Shirley, B. (2002). Biosynthesis of flavonoids and effects of stress. Curr Opin Plant Biol *5*, 218-223.

Woo, H.H., Jeong, B.R., and Hawes, M.C. (2005). Flavonoids: from cell cycle regulation to biotechnology. Biotechnol Lett *27*, 365-374.

Woodward, A.W., and Bartel, B. (2005). Auxin: regulation, action, and interaction. Ann Bot (Lond) *95*, 707-735.

Wu, G., Lewis, D.R., and Spalding, E.P. (2007). Mutations in Arabidopsis multidrug resistance-like ABC transporters separate the roles of acropetal and basipetal auxin transport in lateral root development. Plant Cell *19*, 1826-1837.

Yamagami, M., Haga, K., Napier, R.M., and Iino, M. (2004). Two distinct signaling pathways participate in auxin-induced swelling of pea epidermal protoplasts. Plant Physiol *134*, 735-747.

Yang, H., and Murphy, A.S. (2009). Functional expression and characterization of Arabidopsis ABCB, AUX 1 and PIN auxin transporters in Schizosaccharomyces pombe. Plant J *59*, 179-191.

Yang, Y., Hammes, U.Z., Taylor, C.G., Schachtman, D.P., and Nielsen, E. (2006). High-affinity auxin transport by the AUX1 influx carrier protein. Curr Biol *16*, 1123-1127.

Zazimalova, E., Krecek, P., Skupa, P., Hoyerova, K., and Petrasek, J. (2007). Polar transport of the plant hormone auxin - the role of PIN-FORMED (PIN) proteins. Cell Mol Life Sci *64*, 1621-1637.

Zegzouti, H., Anthony, R.G., Jahchan, N., Bogre, L., and Christensen, S.K. (2006a). Phosphorylation and activation of PINOID by the phospholipid signaling kinase 3-phosphoinositide-dependent protein kinase 1 (PDK1) in Arabidopsis. Proc Natl Acad Sci U S A *103*, 6404-6409.

Zegzouti, H., Li, W., Lorenz, T.C., Xie, M., Payne, C.T., Smith, K., Glenny, S., Payne, G.S., and Christensen, S.K. (2006b). Structural and functional insights into the regulation of Arabidopsis AGC VIIIa kinases. J Biol Chem *281*, 35520-35530.

Zhao, Y., Christensen, S.K., Fankhauser, C., Cashman, J.R., Cohen, J.D., Weigel, D., and Chory, J. (2001). A role for flavin monooxygenase-like enzymes in auxin biosynthesis. Science *291*, 306-309.

Zheng, L.H., Bao, Y.L., Wu, Y., Yu, C.L., Meng, X., and Li, Y.X. (2008). Cantharidin reverses multidrug resistance of human hepatoma HepG2/ADM cells via down-regulation of P-glycoprotein expression. Cancer Lett *272*, 102-109.

Zhou, H.W., Nussbaumer, C., Chao, Y., and DeLong, A. (2004). Disparate roles for the regulatory A subunit isoforms in Arabidopsis protein phosphatase 2A. Plant Cell *16*, 709-722.

Zourelidou, M., Muller, I., Willige, B.C., Nill, C., Jikumaru, Y., Li, H., and Schwechheimer, C. (2009). The polarly localized D6 PROTEIN KINASE is required for efficient auxin transport in Arabidopsis thaliana. Development *136*, 627-636.

8 CURRICULUM VITAE - SEPTEMBER 2009

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Supervisor: Prof. Walter J. Gehring, Biozentrum, Basel

Oct 2000 - April 2005 Studies in Biology at Basel University, Biozentrum, Basel

Scientific Research and Technical Expertise

- **Basic methods in molecular biology and biochemistry:**
  nucleic acid plant tissues and bacteria cells**,** protein extraction from plant tissue, bacteria/yeast cells, PCR, DNA electrophoresis, restriction enzyme digests, cloning of DNA fragments, *southern, western blots* analyses, cDNA library construction and screening, heterologous protein expression and purification in *E. coli*, *in vitro* phosphorylation assay, ATPase activity assay.

- **Yeast molecular genetics and biochemistry:**
  yeast transformation, expression of recombinant proteins, drop tests, transport assay, membrane extraction, binding assay.

- **Plant biology, molecular biology and biochemistry:**
  A. *thaliana* and N. *benthamiana* protoplast preparation and transport assays, *Agrobacterium*-mediated transformation of A. *thaliana* and N. *benthamiana*, transient gene expression in A. *thaliana* mesophyll protoplasts, microsome extraction of a. *thaliana*, confocal microscope analysis of biological specimens, binding assays, A. *thaliana* basic genetics and crossings.

- **Bioinformatics:**
  Routine usage of programs of *Microsoft Office package*. Good expertise in using on-line databases. Good knowledge of bioinformatics tools for nucleotide and amino acidic sequence analysis (BLAST, Clustal, Vector NTI, Genevestigator). *In silico* prediction of phosphorylation sites (PhosphoELM, PhosphoSite, NetPhos 2.0, NetPhosK 1.0, Disphos 1.3, Scansite, The PredictProteinServer), basic skills for Photoshop.

- **Other:**
  In situ hybridization of P. *mammillata* tadpoles.

Courses and Activities during my PhD Studies

Plant Science Center Zurich-Basel

- Scientific Presentation Practice, 1CP
  Penelope Barnett
  22. and 29. August 2006
- Challenges in Plant Science – Colloquium, 2CP
  6. November 2006
- Functional Genomics: Introduction to Transcriptional Profiling and Proteomic, 1CP
  Lars Henning and Sacha Baginsky
  25. and 26. April 2007
- Introduction to Fluorescence Microscopy and Image Processing, 1CP
  Christof Sauter
  16. November, 3. And 12. December 2007
- Elements of a Scientific Career Strategy, 1CP
  Monika Clausen
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- Scientific Organization Committee of the Third International Symposium
  "Plants and People - Mutual Dependence in the 21st century", 3 CP
- Co-coordinator of the Organization Board "Plant Science Center PhD Students Association" ((PS_2)A)
  June 2007 – July 2009

Others:

- Basic Management Skills, 4CP
  Rolf Specht, ETHZ
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- Peer Oberservation,
  Pamela Alean-Kirkpatrick, UZH
  March 2007

Meetings and Presentations during my PhD studies

- Frontiers in Phytohormone Science
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- PSC Symposium "Plant Reproductive Systems"
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  5. May 2008
  Oral Presentation: "Regulation of auxin transport by protein phosphorylation".
- PhD Symposium "Plants and People - Mutual Dependence in the 21st century"
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- 6th International NCCR Symposium on New Trends in Structural Biology
  University of Zurich, Switzerland
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  Glasgow, Scotland
  25.-27. June 2009
  Poster presentation: "PINOID counteractively regulates ABCB/PGP- and PIN- mediated auxin transport". **Henrichs, S.**, Vincenzetti, V., Fukao, Y., Geisler M..
- Society of Experimental Biology (SEB) Meeting 2009
  Glasgow, Scotland
  28. June – 1. July 2009
  Oral Presentation: "PINOID counteractively regulates ABCB/PGP- and PIN- mediated auxin transport".

Publication list

"Flavonoids redirect PIN-mediated polar auxin fluxes during root gravitropic responses."
Santelia D, **Henrichs S**, Vincenzetti V, Sauer M, Bigler L, Klein M, Bailly A, Lee Y, Friml J, Geisler M, Martinoia E.
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